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ENDOCRINOLOGY

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# THE EFFECTS OF HYPOPHYSECTOMY ON THE RESPIRATION AND CELLULAR COMPONENTS OF THE BONE MARROW OF THE RAT[1]

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A LITERATURE is steadily accumulating which indicates that the endocrine system exerts a supportive influence upon certain phases of blood formation. The evidence for this relation is derived in part from the behavior of the formed elements of the blood and the bone marrow histology and cytology in animals experiencing various forms of endocrine deficiency (Gordon and Charipper, 1947; Daughaday, Williams and Daland, 1948). That the hypophysis may represent the initiating source of the endocrine influence on hemopoiesis is indicated by the peripheral anemia and the hypoplasia of the bone marrow which follows removal of this gland (Vollmer, Gordon, Levenstein and Charipper, 1939; Crafts, 1941). Moreover, the administration of principles of endocrine organs regulated by the adenohypophysis results in the amelioration of the hemic dysfunctions induced by hypophyseal removal (Meyer, Thewlis and Rusch 1940; Vollmer and Gordon, 1941; Vollmer, Gordon and Charipper, 1942; Crafts, 1946 a, b).

To arrive at a clearer understanding of the fundamental mechanisms underlying bone marrow function it seems inevitable that research on this organ must be directed at a cellular metabolic level. Techniques have already been established for studying the respiratory metabolism of bone marrow under a variety of experimental conditions (v. Bréza, 1926; Warren, 1934, 1940 a, b, 1941, 1942; Bock and Felix, 1939, 1940; Goldinger,

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Lipton and Barron, 1947; Bird and Evans, 1949). To our knowledge, however, no experiments have been performed as yet to determine the respiratory metabolism of the bone marrow of animals subjected to endocrine deficiencies. The present work will describe the effects of hypophysectomy upon the oxygen consumption of rat bone marrow and the relation of the changes to the marrow cytologic alterations induced by this operation.

## METHODS

Adult male hypophysectomized rats and intact litter mate controls of the Sprague-Dawley strain were used. The animals were approximately 2 months old and weighed about 150 gm. at the time of operation. The rats were maintained on a standard laboratory ration fortified with milk, bread, canned and fresh meat, and fresh vegetables. Experiments were conducted at 2 1/2 to 4 months after hypophysectomy. Completeness of hypophyseal removal was established by a careful examination of the sella turcica contents and by target endocrine organ weights at the time of sacrifice.

The neutralized serum technique of MacLeod and Rhoads (1939) was used for the respiratory studies, since it has been shown by Warren (1940 a,b) that bone marrow in this medium displays a more normal type of metabolism than in Ringer's solution. To reduce the time interval between the death of the animal and the start of readings, serum obtained from similar rats of a preceding experiment was used. The animals were anesthetized lightly with ether and exsanguinated by heart puncture. The blood was allowed to clot at room temperature, stored overnight in a refrigerator to permit clot retraction and the serum separated the next day. This was stored in the frozen state for use in the subsequent experiment. Immediately upon blood withdrawal, the femora and tibiae were removed, scraped clean of adhering muscle, cracked open, the bone marrow removed and placed immediately upon a watch glass in a fluid medium prepared in the following manner: 0.1 ml. of 0.1 N HCl was added to each ml. of serum in order to bring the pH to 6.6–6.8. The acidified serum was evacuated by a water suction pump to remove the carbon dioxide. 0.1 ml. of M/10 $Na_2HPO_4$ buffer (pH 7.4) was then added to each ml. of serum. Finally, 0.5 ml. of Krebs-Ringer solution was added to each ml. of serum. The addition of the Krebs-Ringer was adopted since the volume of serum available from each hypophysectomized rat was generally insufficient for the experiment.

A modification of the marrow suspension technique introduced by Warren (1942) was employed in the respiratory studies. The marrow was minced with a fine scissors, transferred to a 15 ml. graduated centrifuge tube, and a fine suspension formed by drawing the material up and down in a 1 ml. tuberculin syringe through a 20 gauge needle. This suspension was then further diluted with the neutralized serum, and smears made for determination of the myelogram. One ml. of the suspension was removed for total nitrogen determinations and 1.0 ml. was placed in each of two 5 ml. Warburg flasks. These contained 0.2 ml. 10% KOH and #40 Whatman filter paper in the center well to absorb the $CO_2$ and provide direct measurements of the $O_2$ uptake (Umbreit, Burris and Stauffer, 1949). The flasks were attached to standard Warburg manometers, placed in the constant temperature bath (37.0° C.) and shaken 105 three cm. strokes per minute for a temperature equilibration period of 15 minutes. Stopcocks were then closed and readings started. The average interval of time between the death of the animal and initial readings was 50 minutes. Readings were taken every 10 minutes over a 3 hour period.

The determinations of the total nitrogen were made in duplicate 0.5 ml. aliquots. The

serum in the 2 samples was removed by centrifugation and the marrow cells were washed with ice-cold Krebs-Ringer solution. The suspension was centrifuged and washed 3 additional times to remove serum traces. Nitrogen determination on these washed cells was made by the "long method" of Miller and Miller (1948), a colorimetric micro-Kjeldahl procedure.

Oxygen uptake is given as $QO_2(N)$ (i.e. $\mu$l. $O_2$/mg. N/hr.). The nitrogen basis is used in preference to dry weight because of the variable fat content of marrow tissue.

To provide the same environment for the respiring tissue as within the body, the metabolism of normal marrow was measured in normal serum and that from hypophysectomized animals in the serum of similarly-operated rats. Some experiments were also conducted on normal marrow in the serum of hypophysectomized rats. For each sample, the oxygen uptake of the serum itself was determined since it has been shown (MacLeod and Rhoads, 1939) that as much as 15% of the total consumption is due to serum. In all cases the oxygen consumption figures are given in terms of the total values minus that of the serum alone.

The marrow smears were stained with Wright or Giemsa. When the latter was used, a 1:50 dilution buffered at pH 5.8 (Endicott, 1945) gave the best differentiation. 1500 nucleated cells were counted for each animal and classified under the headings suggested by Endicott and Ott (1945). In addition, 2000 cells were examined to determine the percentage of mature non-nucleated erythrocytes in the total cellular content of each sample.

## RESULTS

### 1. *Marrow myelograms*

Observation of Table 1 indicates that hypophysectomy results in a decrease in the percentages of immature neutrophilic granulocytic forms in the bone marrow. The ratio of the more mature to younger neutrophilic

TABLE 1. MEAN VALUES (%) ±STANDARD ERRORS OF NORMAL AND HYPOPHYSECTOMIZED RAT BONE MARROW CELL TYPES

| | *Hypophysectomized* (8 animals) | *Controls* (7 animals) | $P$[1] |
|---|---|---|---|
| Blasts | 0.8±0.32 | 0.7±0.11 | >0.6 |
| Eosinophiles | 4.1±0.63 | 3.2±0.37 | >0.2 |
| Neutrophilic granulocytes | | | |
| Promyelocytes & myelocytes | 3.0±0.82 | 6.6±0.43 | <0.01 |
| Metamyelocytes & segmented nuclear forms | 25.3±1.64 | 23.0±1.82 | >0.2 |
| Total granulocytes | 32.4±2.06 | 32.8±2.07 | >0.8 |
| Lymphocytes | 9.1±0.83 | 6.5±0.64 | <0.02 |
| Total nucleated erythroid cells | 55.7±1.78 | 59.4±1.81 | >0.10 |
| Total nucleated cells (erythroid +myeloid) | 69.3±4.32 | 58.6±2.52 | <0.05 |
| Erythroid-myeloid cell ratio | 1.78±0.14 | 1.85±0.45 | >0.8 |
| Miscellaneous | 1.1±0.11 | 0.6±0.14 | <0.01 |

[1] P is calculated from the distribution of Fisher's t. Values <0.05 are considered significant.

cell forms in the bone marrow of normal rats is approximately 3.5 whereas it is about 8.4 for marrow of hypophysectomized rats. In 2 long-term hypophysectomized rats, the combined promyelocytic and myelocytic values amounted to only 0.4% and 0.7%. Actually, promyelocytes are

noted only rarely in the marrows of hypophysectomized rats. The mean values for percentages of neutrophilic metamyelocytic and segmented nuclear forms are not affected significantly by hypophyseal removal. The older segmented forms, however, although not classified as a separate category, appear to be more numerous in hypophysectomized rats.

Lymphocytic percentages are elevated significantly by hypophyseal removal whereas eosinophilic cell percentages are unaffected. The concentration of total nucleated erythroid cells and the erythroid-myeloid cell ratics likewise remain uninfluenced by pituitary removal.

Total nucleated cell percentages (erythroid plus myeloid) are significantly higher in the marrow tissue of hypophysectomized than in normal rats.

2. *Marrow respiration*

It will be noted from Figure 1 that, when based on total nitrogen concentration of the entire sample, the $QO_2$ values for the marrow of hypophy-

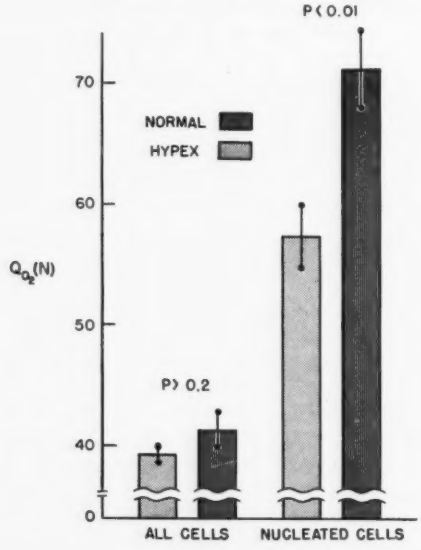


FIG. 1. $QO_2(N)$ values for bone marrow of 8 hypophysectomized and 7 control rats. Vertical lines drawn through the tops of the bars represent $\pm 1$ Standard Error of the Mean.

sectomized rats are not significantly different from those of normal rats. If, however, the percentages of mature red blood cells in the marrow smears are taken into consideration and calculation is made on the basis of nucleated cells only (i.e. by dividing the $QO_2(N)$ by the percentage of

nucleated cells for each animal), a significant difference between the experimental and control values is revealed.

Two experiments were carried out to compare the respiration of bone marrow in normal and hypophysectomized rat serum. No difference was noted in the ability of the two sera to support the respiratory metabolism of normal marrow.

The values indicated in Figure 1 are those obtained during the first hour in the respirometers. At the end of the second hour, the respiration of normal marrow had decreased to approximately 90% and by the end of the third hour to 80% of the first hour values. For the hypophysectomized animals, second hour readings averaged 82%, and the third hour values 75% of those obtained for the first hour.

## DISCUSSION

The results indicate that, provided the calculations are made on the basis of nucleated cell content, the bone marrow of hypophysectomized rats displays a lower $QO_2$ than normal rat marrow. The justification of attributing the respiration of bone marrow to its nucleated cells is derived from the finding by Ramsey and Warren (1932) that the $QO_2$ of mature erythrocytes is very low, being about 1/200 that of the marrow as a whole. Thus these cells, while contributing to the nitrogen content of the sample, consume so little oxygen that they can best be discounted from the $QO_2$ calculations. It is probable that the higher percentages of total nucleated cells detected in the bone marrow suspensions of hypophysectomized rats are due to admixture with peripheral blood which contains a smaller number of red cells than that found in normal animals. The peripheral red cell counts of the hypophysectomized animals used in this study averaged about 8 million per cu. mm. whereas those of unoperated litter mate controls were approximately 10 million per cu. mm.

The oxygen uptake values in this report are given in terms of the total nitrogen content of the washed cells. The total nitrogen of bone marrow has been estimated in one instance as 14.6% (Orr and Stickland, 1938) and in another as 11.42% (Goldinger, Lipton and Barron, 1947) of the fat-free dry weight. These figures are for rabbit bone marrow and may not apply to the marrow of intact or hypophysectomized rats. Likewise the factor 6.25 used by Bird and Evans (1949) to convert total nitrogen to protein values for rabbit bone marrow may not hold for the hypophysectomized animal. It was decided, therefore, to adopt the procedure of relating the $QO_2$ values to total nitrogen of the sample as such since this was the only direct chemical measurement of the marrow cells made in this study.

The present finding that a decrease in the percentages of neutrophilic

promyelocytes and myelocytes in the marrow occurs after hypophysectomy is in agreement with results reported previously by Arvy, Gabe and Stutinsky (1948) who also employed bone marrow smears for their determinations. The differential counts made on bone marrow sections by Crafts (1946 a, b) do not show this change in the granulocytic precursors but reveal a decrease in the percentages of total erythroid elements which was not observed in the present study. While sections were not employed for making marrow counts in our work, it was noted that when clumps of cells were encountered in smears, it became necessary to count at least 1500 cells before a reliable figure was obtained. Counts of 500 cells in sections therefore may not be representative of the cellular pattern of the entire marrow. Another criticism of utilizing sections is that values obtained in the areas counted may not necessarily be the same at other levels of the marrow not included in the analysis.

The development of a peripheral anemia in the hypophysectomized rat despite the maintenance of normal erythroid cell percentages within the marrow may be explained in a number of ways. It has been shown that there is extensive fat infiltration and a reduction of total marrow mass in the hypophysectomized rat (Vollmer, Gordon, Levenstein and Charipper, 1939). Thus the numbers of cells released into the circulation per unit time might be decreased. The additional possibility that a decreased rate of development throughout the marrow erythroid series also contributes to this phenomenon would have to be tested.

At the present, one can only speculate on the relation of the drop in $QO_2(N)$ in the marrow of the hypophysectomized rat to the alterations induced in its cellular composition. It is generally agreed that although the cells of the erythroid series are characterized by a moderately high $QO_2$, and the white cell metabolism is predominatly glycolytic, the myeloid cells also contribute significantly to the oxidative metabolism of marrow (Warren, 1934, 1940 b; Bock and Felix, 1940). The decrease in the ratio of immature to mature neutrophilic myeloid elements and the rise in lymphocytic percentages noted in the bone marrow of the hypophysectomized animal might provide the explanation for the drop in $QO_2$. It has been reported also (Arvy, Gabe and Stutinsky, 1948) that an increase in the percentages of marrow normoblasts and a decrease in proerythroblasts follow pituitary removal in the rat. Although no attempt was made in the present myelogram studies to delineate the earlier erythroid forms (proerythroblasts) from the more mature ones (erythroblasts and normoblasts), a change in this ratio might also constitute the basis for the alteration in respiratory metabolism noted in hypophysectomized rats. Warren (1934) and Bock and Felix (1940) have found, in fact, a direct relation between the extent of erythroid cell immaturity in marrow and its

oxygen consumption. A study of the respiration of pure marrow cellular types, if they could be obtained, would help greatly in resolving the mechanisms underlying the influence of the endocrine system on marrow metabolism. The possibility cannot be precluded also that, apart from the alterations induced by hypophysectomy in the types of elements, the entire marrow cellular population suffers a drop in oxygen consumption as do other tissues of the body in the absence of the hypophyseal hormones. A more complete understanding of the metabolism of the bone marrow in hypophysectomized animals could undoubtedly be obtained through an examination of the glycolytic and enzymatic behavior in this tissue. Some aspects of this problem are being investigated currently in our laboratories.

## SUMMARY

Hypophysectomy in the rat results in a decrease in the percentages of neutrophilic promyelocytes and myelocytes and an increase in the concentration of lymphocytic forms in the bone marrow. The percentages of total eosinophilic cells, total nucleated erythroid elements and the erythroid-myeloid cell ratios are unaffected by hypophyseal removal. Total nucleated cell percentages (erythroid plus myeloid) are higher in bone marrow suspensions of hypophysectomized than in normal rats.

The rate of oxygen consumption, when based on total nitrogen concentration of the entire marrow sample, is similar in hypophysectomized and unoperated rats. When related to nucleated cell content only, the $QO_2(N)$ values for bone marrow of hypophysectomized animals are significantly lower than those for normal rats. Alterations in the ratio of immature to more mature cellular components may provide the explanation for the change in $QO_2(N)$ observed for the nucleated cells of the marrow of hypophysectomized rats.

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# DIFFERENCES IN THE RESPONSE OF INBRED LINES OF MICE TO THIOURACIL AND THYROPROTEIN[1]

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IT IS well known that there are wide species, sex and age differences in thyroid activity; that marked alteration in thryoid function can be brought about by environmental changes such as temperature; and that alteration of nutritional or endocrine status may affect the activity of the thyroid gland. Less well known are the influences of genetic constitution on thyroid function. Such differences in genetic makeup may be referred to as breed differences within a species, e.g., differences between the White Leghorn, Rhode Island Red, etc. Strain differences, in the case of chickens, generally refer to chickens which have been selected over a period of time for a specific purpose such as rapid-growth, slow-growth, rapid-feathering, etc. Line differences, as in mice, generally refer to lines of mice which have been selected over a period of time for some particular character such as growth rate, mature body size, etc.

Boettiger (1941) showed that the oxygen consumption of a strain of dwarf mice was 30 to 40% below that of normal mice of similar weight and that the administration of appropriate amounts of thyroxine would increase oxygen consumption to the levels of normal mice. Koger *et al.* (1942) reported that in virgin female mice of unknown ancestry crystalline thyroxine increased food intake and rate of growth, increased the water and nitrogen content of the carcasses and decreased the amount of fat. Hurst and Turner (1948) found differences in thyroxine secretion rate, growth rate and mature body size between the Schwing, $C_3H$, Rockland all purpose albino, and a yellow strain of mice.

Several investigators have reported breed and strain differences in thyroid activity of chickens. Schultze and Turner (1945) found that the thyroxine secretion rate per unit of body weight was greater in White Leghorn than in White Plymouth Rock cockerels. Mixner and Upp (1947) found that the hybrid chicks which resulted from crossing four inbred

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[2] The authors express their appreciation to Doctors E. J. Warwick and W. L. Lewis for the lines of mice, to Dr. K. K. Chen of the Lilly Research Laboratories for the thiouracil and to Dr. W. R. Graham, Jr. of Cerophyl Laboratories for the thyroprotein.

lines of two breeds had a higher thyroxine secretion rate than single crossed chicks or ordinary intrabreed crossbreds. They suggested that increased thyroid activity might be a factor in heterosis or hybrid vigor. Glazener, Shaffner and Jull (1949) measured thyroxine secretion rate, and the influence of thyroprotein on growth, feed efficiency and rate of feathering in rapid- and slow-growing strains of New Hampshire and Barred Plymouth Rock chickens. Thyroxine secretion rate, measured only in the females, was higher in the rapid-growing strains. The feeding of thyroprotein depressed growth in the rapid-growing New Hampshires and stimulated growth in the slow-growing New Hampshires and rapid-growing Barred Rocks. It was concluded that each strain had "a physiological optimum level of thyroid activity for growth, feed utilization and feathering."

The purpose of this study was to determine the effects of thiouracil and thyroprotein upon growth, thyroid weight and histology and testis weight and histology in seven inbred lines of mice which had been selected for differences in mature body size.

## PROCEDURE

The population from which the various lines of mice were derived was obtained from Dr. J. W. MacArthur of the University of Toronto in February, 1948. These mice were first interbred by Doctors E. J. Warwick and W. L. Lewis at Purdue in order to increase genetic variability as much as possible and were then selected for Large, Medium and Small body size for five generations. After five generations the percent of inbreeding of the various lines ranged from 40 to 52%. The average 60 day weights of the lines were as follows: Large—28.35 gm.; Medium—19.50 gm.; Small—14.65 gm. Two lines of mice were used in each size group. In Trial 2, one of the lines of Large mice did not reproduce satisfactorily and another line was substituted for it.

The mice were fed a commercial poultry mash which contained 20% crude protein, 2.5% crude fat and not more than 8.0% crude fiber. Thyroprotein was added to the ration at a rate of 10 gm. per 100 pounds of feed (0.022%). Thiouracil was added to the ration at levels of 22.7 gm. and 68.1 gm. per 100 pounds of feed (0.05% and 0.15%) respectively.

The mice were fed the control or experimental rations from 21 until 60 days of age, at which time the animals were killed and examined. Records were obtained of body weight, thyroid and gonad weights and histology. Four mice of each line, sex, and size group were assigned to each treatment at 21 days of age. Litter mates were used whenever possible. In the second trial, because of smaller litter size, it was occasionally necessary to use

three mice of a particular line, sex or size group. A total of 192 mice were used in Trial 1 and 175 in Trial 2.

Carcass analyses of 10 randomly selected, non-treated mature mice of each sex were made in order to determine if the lines of mice varied in chemical composition. Each mouse was skinned and eviscerated and a composite sample of the 10 mice was obtained by grinding them in a food mill. Analyses were made for nitrogen, fat and moisture by standard procedures.

The various data were treated statistically by the method of analysis of variance.

## RESULTS

The effects of thyroprotein and thiouracil on body weight at 60 days of age are summarized in Tables 1 and 2. With the exception of Large line 3,

TABLE 1. THE EFFECTS OF THYROPROTEIN AND THIOURACIL ON BODY WEIGHT (IN GRAMS) AT 60 DAYS OF AGE. TRIAL 1

| Lines | Treatment | | | |
|---|---|---|---|---|
| | Control | 0.022% Thyroprotein | 0.05% Thiouracil | 0.15% Thiouracil |
| *Large* | | | | |
| Line 1 | | | | |
| Males | 31.75 | 28.25† | 29.25 | 26.75† |
| Females | 25.50 | 23.25 | 24.75 | 24.00 |
| Line 2 | | | | |
| Males | 32.25 | 27.25† | 30.50 | 28.00* |
| Females | 24.50 | 23.75 | 22.25 | 22.00 |
| *Medium* | | | | |
| Line 4 | | | | |
| Males | 23.00 | 22.25 | 21.75 | 19.75 |
| Females | 17.00 | 19.00 | 17.25 | 15.75 |
| Line 5 | | | | |
| Males | 20.25 | 22.25 | 19.75 | 18.00 |
| Females | 17.75 | 18.50 | 16.75 | 17.00 |
| *Small* | | | | |
| Line 6 | | | | |
| Males | 14.50 | 15.25 | 15.50 | 13.50 |
| Females | 11.50 | 13.50 | 11.50 | 9.50 |
| Line 7 | | | | |
| Males | 18.25 | 17.75 | 15.75† | 15.50† |
| Females | 14.25 | 16.00 | 14.25 | 12.75 |

* Significantly different than the controls ($P < 0.05$).
† Highly significantly different than the controls ($P < 0.01$).

which was used only in Trial 2, thyroprotein consistently reduced rate of growth as measured by body weight in the Large lines. In the Medium and Small lines, when the sex-groups were considered separately, body weight was increased in 14 of the 16 groups of mice. These differences were statistically significant in only one group, but the consistency of the trend is clear. Thiouracil fed at levels of either 0.05% or 0.15% reduced growth

rate in the Large lines of mice in both trials. As shown in the tables, statistical significance was confined to the 0.15% level of thiouracil with one exception. In the Medium and Small lines 0.05% thiouracil had no significant effect on 60 day weight except in the males of line 7 in Trial 1. However, the fact that line 7 was not significantly affected in Trial 2, justifies the conclusion that this level of thiouracil had little effect on the weight in the Medium or Small lines. While all groups receiving 0.15% thiouracil showed reduced growth, only 3 of the 16 differed significantly.

TABLE 2. THE EFFECTS OF THYROPROTEIN AND THIOURACIL ON BODY WEIGHT (IN GRAMS) AT 60 DAYS OF AGE. TRIAL 2.

| Lines | Treatment | | | |
|---|---|---|---|---|
| | Control | 0.022% Thyroprotein | 0.05% Thiouracil | 0.15% Thiouracil |
| *Large* | | | | |
| Line 2 | | | | |
| Males | 31.50 | 29.25 | 30.25 | 29.75 |
| Females | 25.66 | 26.33 | 23.33* | 23.66* |
| Line 3 | | | | |
| Males | 30.33 | 33.33 | 30.33 | 28.00† |
| Females | 25.33 | 27.00 | 24.33 | 24.00 |
| *Medium* | | | | |
| Line 4 | | | | |
| Males | 20.25 | 22.25 | 21.75 | 19.50 |
| Females | 19.50 | 19.75 | 16.75 | 18.00 |
| Line 5 | | | | |
| Males | 20.00 | 21.00 | 20.00 | 19.00 |
| Females | 18.25 | 19.75 | 18.50 | 16.00* |
| *Small* | | | | |
| Line 6 | | | | |
| Males | 16.00 | 17.25 | 14.66 | 15.25 |
| Females | 13.25 | 14.25 | 13.00 | 11.66 |
| Line 7 | | | | |
| Males | 15.50 | 17.75* | 16.50 | 14.00 |
| Females | 14.00 | 15.25 | 13.75 | 12.50 |

* Significantly different than the controls ($P < 0.05$).
† Highly significantly different than the controls ($P < 0.01$).

The effects of thyroprotein and thiouracil on gonad and thyroid weights are summarized in Tables 3 and 4. In both trials gonad weight expressed as per cent of body weight was significantly greater ($P < 0.01$) in the Medium and Small lines than in the Large lines. This was true in the treated as well as the control groups. Thyroprotein produced no significant alteration of gonad weight in either sex. Thiouracil at levels up to 0.15% of the ration, had no significant effect on either testis or ovarian weights or histology.

With an occasional exception, thyroid weight was greater in the thiouracil treated females than in the males. This sex difference was significant ($P < 0.05$) in the Small size group and ($P < 0.01$) in the Medium and Large

TABLE 3. THE EFFECTS OF THYROPROTEIN AND THIOURACIL ON THYROID AND GONAD WEIGHT OF MICE. EXPRESSED AS PER CENT OF BODY WEIGHT. TRIAL 1

| | Treatment | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Control | | 0.022% Thyroprotein | | 0.05% Thiouracil | | 0.15% Thiouracil | |
| Lines | Gonads | Thyroids | Gonads | Thyroids | Gonads | Thyroids | Gonads | Thyroids |
| *Large* | | | | | | | | |
| Line 1 | | | | | | | | |
| Males | .64 | .013 | .68 | .012 | .70 | .027 | .82 | .052 |
| Females | .052 | .016 | .030 | .016 | .039 | .039 | .040 | .063 |
| Line 2 | | | | | | | | |
| Males | .70 | .018 | .73 | .013 | .70 | .056 | .75 | .074 |
| Females | .068 | .026 | .048 | .011 | .060 | .064 | .062 | .085 |
| *Medium* | | | | | | | | |
| Line 4 | | | | | | | | |
| Males | .88 | .010 | 1.07 | .010 | 1.04 | .052 | 1.11 | .066 |
| Females | .077 | .014 | .074 | .011 | .059 | .049 | .070 | .078 |
| Line 5 | | | | | | | | |
| Males | 1.04 | .019 | .87 | .010 | 1.06 | .074 | 1.00 | .128 |
| Females | .042 | .021 | .028 | .028 | .044 | .093 | .046 | .108 |
| *Small* | | | | | | | | |
| Line 6 | | | | | | | | |
| Males | .93 | .014 | .95 | .014 | .96 | .044 | 1.04 | .129 |
| Females | .074 | .013 | .076 | .010 | .063 | .067 | .038 | .138 |
| Line 7 | | | | | | | | |
| Males | .93 | .012 | .86 | .015 | .85 | .031 | .92 | .104 |
| Females | .044 | .020 | .054 | .010 | .042 | .062 | .032 | .106 |

TABLE 4. THE EFFECTS OF THYROPROTEIN AND THIOURACIL ON THYROID ANE GONAD WEIGHT OF MICE. EXPRESSED AS A PER CENT OF BODY WEIGHT. TRIAL 2

| | Treatment | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Control | | 0.022% Thyroprotein | | 0.05% Thiouracil | | 0.15% Thiouracil | |
| Lines | Gonads | Thyroids | Gonads | Thyroids | Gonads | Thyroids | Gonads | Thyroids |
| *Large* | | | | | | | | |
| Line 2 | | | | | | | | |
| Males | .64 | .020 | .67 | .017 | .61 | .051 | .66 | .071 |
| Females | .047 | .022 | .076 | .013 | .059 | .058 | .066 | .082 |
| Line 3 | | | | | | | | |
| Males | .78 | .013 | .73 | .008 | .75 | .040 | .80 | .073 |
| Females | .041 | .010 | .056 | .011 | .048 | .066 | .040 | .080 |
| *Medium* | | | | | | | | |
| Line 4 | | | | | | | | |
| Males | 1.13 | .024 | .97 | .020 | .99 | .061 | 1.01 | .071 |
| Females | .057 | .022 | .096 | .021 | .067 | .071 | .067 | .079 |
| Line 5 | | | | | | | | |
| Males | 1.19 | .028 | 1.12 | .021 | .81 | .069 | 1.24 | .124 |
| Females | .070 | .030 | .070 | .020 | .062 | .116 | .057 | .156 |
| *Small* | | | | | | | | |
| Line 6 | | | | | | | | |
| Males | 1.18 | .019 | 1.00 | .015 | 1.00 | .063 | 1.18 | .117 |
| Females | .055 | .027 | .100 | .020 | .068 | .092 | .056 | .108 |
| Line 7 | | | | | | | | |
| Males | 1.00 | .020 | .92 | .022 | .97 | .075 | 1.11 | .100 |
| Females | .058 | .032 | .049 | .018 | .044 | .106 | .042 | .119 |

size groups. In Trial 2, the line-treatment interaction was significant in only the Medium size groups ($P < 0.01$).

Microscopic examination of the thyroid revealed hypertrophy and hyperplasia of the follicular epithelium in the thiouracil-treated mice and a slight reduction in cell height of the thyroprotein treated mice as compared with the controls.

The carcass analyses of the untreated mice from the various lines are

TABLE 5. CARCASS ANALYSES OF REPRESENTATIVE GROUPS OF 10 MICE EACH FROM 7 INBRED LINES OF MICE

| Lines | Component percentages | | |
|---|---|---|---|
| | Fat | Protein | Moisture |
| *Large* | | | |
| Line 1 | | | |
| Males | 14.49 | 17.28 | 62.94 |
| Females | 10.32 | 17.84 | 65.60 |
| Line 2 | | | |
| Males | 9.62 | 18.34 | 65.90 |
| Females | 7.93 | 19.28 | 67.81 |
| Line 3 | | | |
| Males | 6.86 | 19.50 | 68.31 |
| Females | 7.23 | 18.24 | 67.16 |
| *Medium* | | | |
| Line 4 | | | |
| Males | 6.46 | 19.93 | 69.40 |
| Females | 6.76 | 19.47 | 67.44 |
| Line 5 | | | |
| Males | 6.41 | 20.14 | 68.56 |
| Females | 7.53 | 19.06 | 66.45 |
| *Small* | | | |
| Line 6 | | | |
| Males | 5.07 | 20.00 | 69.22 |
| Females | 4.88 | 19.70 | 68.85 |
| Line 7 | | | |
| Males | 6.64 | 19.12 | 67.58 |
| Females | 7.38 | 19.44 | 67.67 |

summarized in Table 5. Since composite samples of tissue were used, statistical analyses of these data were not applicable. An inspection of the data indicates, however, that Large lines 1 and 2 were more obese than any other lines.

## DISCUSSION

The results of these studies furnish additional evidence of physiological and endocrine differences between lines or strains of the same species and support the conclusions of Boettiger (1941), Koger *et al.* (1942) and Hurst and Turner (1948) that strains of mice differ in thyroid physiology.

From the genetic standpoint they emphasize the fact that selection for a factor such as body size as determined by weight may result in quite dissimilar endocrine makeup. The Small and Medium sized lines were active

and highly exitable whereas the Large lines were quite docile in behavior. This observation might suggest that the thyroid activity was less in the Large lines of mice. However, the Large lines, although of a very similar body weight, differed markedly in composition from each other. Large lines 1 and 2 were more obese than Large line 3 or than any of the Medium or Small lines. This suggests again that Large lines 1 and 2 might be hypothyroid. It might be expected that thyroprotein would increase growth rate in hypothyroid lines. An increase in 60 day weight was obtained only in line 3 of the Large group, but thyroprotein consistently increased body weight in all Medium and Small lines. Thiouracil, at levels of either 0.05% or 0.15% of the ration consistently reduced 60 day weight in the Large lines but 0.05% thiouracil had no significant effect on body weight in the Medium or Small lines except in the males of line 7 in Trial 1. It was also observed that thyroid weight, expressed as a percentage of body weight, tended to be greater in the thiouracil treated Medium and Small lines. However, the thyroid weights of the untreated Medium and Small size mice tended to be heavier than those of the Large mice, particularly in Experiment 1. The difference between experiments in this respect might be explained by differences in environmental temperature since the first experiment was conducted between June 13 and July 25, 1950 and the second between June 27 and August 19.

It appears that in selection for body size based on 21 and 60 day weights some of the larger mice may be so classified because of increased fat deposition, Lines 1 and 2. It is interesting to note that in the most obese line (1) it was impossible to repeat the trial because of very poor fertility in the line. Whether the decreased fertility is associated with hypothyroidism, obesity or other endocrine disturbances is a matter for speculation.

## SUMMARY

The effects of thyroprotein and thiouracil upon growth, thyroid weight and histology and gonad weight and histology were studied in 367 mice representing 7 inbred lines and three size groups.

Thyroprotein fed at 0.22% of the diet produced significant increases in body weight in some lines and a significant depression of weight in other lines. Thiouracil fed at a level of 0.15% of the diet consistently reduced body weight in all lines. The administration of thiouracil at a 0.05% level depressed growth in the Large lines but had no significant effect in the Medium or Small lines.

There were highly significant interactions between body size and treatment, sex and treatment and line and treatment with respect to thyroid weight. Thyroprotein and thiouracil had no consistent effect on gonad weight and histology.

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# THE NATURE OF THE CONJUGATED ESTRONE IN HUMAN PREGNANCY URINE[1]

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ESTRIOL, the most abundant of the estrogens in human pregancy urine, has been shown to be largely present as sodium estriol monoglucuronidate (Cohen and Marrian, 1936; Cohen, 1950a). The isolation of estrone sulfate from pregnant mares' urine by Schachter and Marrian (1938) led to the general supposition that the estrone conjugate of human pregancy urine was also a sulfate. Support for this concept has come from two groups of workers. Butenandt and Hofstetter (1939) claimed that a similarity in the properties of concentrates of the human urine estrone conjugate and of estrone sulfate indicated that the two conjugates were indeed identical. Cohen and Bates (1949) reported that a large percentage of both the estrone-estradiol and estriol fractions of human pregnancy urine were freed from their conjugates by a preparation of phenolsulfatase, Mylase P. This preparation was devoid of glucuronidase activity as measured by the procedure of Fishman *et al.* (1948). The experiments described in this paper were devised to obtain further evidence with respect to the nature of the estrone conjugate in human pregnancy urine. While an isolation of this complex was not effected, evidence based on (A) the application to human urine of a procedure devised to concentrate estrone sulfate, (B) a comparison of the distribution of sodium estrone sulfate and of the urinary estrone conjugate between butanol and various aqueous solutions and (C) the hydrolysis of human pregnancy urine with preparations of spleen glucuronidase, tend to indicate that a major portion of the estrone in human pregnancy urine is conjugated as the glucuronide.

## METHODS

*Materials.* All of the pregnancy urine used in these studies was pooled urine and was obtained from patients during the 32nd to 36th week of pregnancy. The male urine used

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[2] The data in this paper were obtained in large part from a thesis presented by Irving B. Oneson to the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

in some of the control experiments was also from pooled batches. All urine specimens were stored in the ice chest and used within 72 hours after collection.

The sodium estrone sulfate employed in the model experiments was kindly supplied by Dr. S. Cook of Ayrest, McKenna and Harrison, Ltd.

*Fractionation and Assay of Estrogen.* The estriol and estrone-estradiol fractions of the hydrolyzed urines were approximately separated by the procedure of Cohen and Marrian (1934) and the fractions assayed by the colorimetric procedure of Stevenson and Marrian (1947). Assays of solutions containing pure estrone sulfate were done without preliminary hydrolysis. In view of the fact that most of the estrogen in the estrone-estradiol fraction of human pregnancy urine is estrone (Stimmel, 1946; Engel, 1950), separation was not attempted for the routine assays and such fractions will be referred to as "estrone" fractions in this paper.

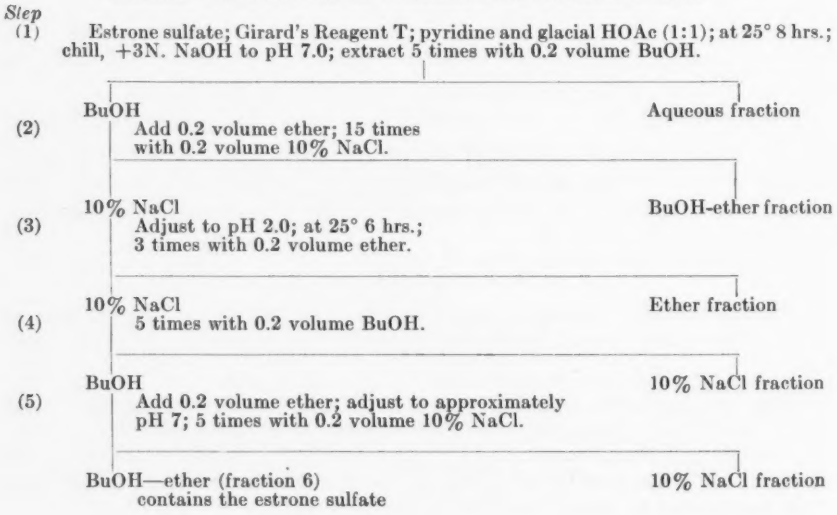
*Hydrolysis of the Conjugated Estrogens.* Hydrolysis with hydrochloric acid was effected by the procedure of Stevenson and Marrian (1947). Hydrolysis of urines with concentrates of calf-spleen glucuronidase was carried out essentially by the method of Cohen (1951).

## EXPERIMENTAL

### A. *Attempted Concentration of Estrone Sulfate from Human Pregnancy Urine. A Modified Girard Procedure for the Concentration of Estrone Estrone Sulfate*

A modification of the Girard procedure (1936) for the separation of ketonic from non-ketonic steroids was devised to apply for estrone sulfate and is shown schematically in Diagram 1. Modifications in the routine Girard procedure centered around the necessity of employing relatively

DIAGRAM 1. MODIFIED GIRARD PROCEDURE FOR ESTRONE SULFATE


Step
(1)
Estrone sulfate; Girard's Reagent T; pyridine and glacial HOAc (1:1); at 25° 8 hrs.; chill, +3N. NaOH to pH 7.0; extract 5 times with 0.2 volume BuOH.
BuOH
Aqueous fraction
(2)
Add 0.2 volume ether; 15 times with 0.2 volume 10% NaCl.
10% NaCl
BuOH-ether fraction
(3)
Adjust to pH 2.0; at 25° 6 hrs.; 3 times with 0.2 volume ether.
10% NaCl
Ether fraction
(4)
5 times with 0.2 volume BuOH.
BuOH
10% NaCl fraction
(5)
Add 0.2 volume ether; adjust to approximately pH 7; 5 times with 0.2 volume 10% NaCl.
BuOH—ether (fraction 6) contains the estrone sulfate
10% NaCl fraction

mild acid conditions in the formation and hydrolysis of the Girard complex, so as to minimize the hydrolysis of the sulfate ester. The essential features of the adopted scheme (Diagram 1) are indicated in the following steps:

(1) The partial neutralization of the acetic acid with pyridine (to give a final pH of 4.5) and the carrying out of the Girard reaction at room temperature permitted for a quantitative formation of hydrazone (as measured with free estrone) and prevented the hydrolysis of estrone sulfate.

The conjugated steroids including those which had formed a hydrazone were removed from the aqueous solution by extraction with butanol.

TABLE 1

| Sample | Starting residue | NaES added | Urinary Estrogen | | Estrogen in final BuOH-ether (fraction 6, diagram I) | | % recovery of added NaES |
|---|---|---|---|---|---|---|---|
| | | | Estriol | "Estrone" | Estriol | Estrone" | |
| | | mg. | mg. | mg. | mg. | mg. | |
| I | NaES | 5.6 | — | — | — | 3.6 | 64 |
| II | Male Urine* (Batch NMU 153) | 1.9 | — | — | — | 1.1 | 58 |
| IIIA | Pregnancy Urine (Batch LPU 135) | — | 8.2 | 0.9 | 1.0 | 0.1 | — |
| IIIB | Pregnancy Urine (Batch LPU 135) | 2.9 | — | — | 1.0 | 1.6 | 52 |

* See text for the details of the preparation of the urine residues used in these experiments.

(2) The addition of ether to the butanol extracts and its extraction with a salt solution permitted an effective separation of the estrone sulfate hydrazone complex from estrone sulfate. About 80–85% of the former material appears in the aqueous extract while estrone sulfate is almost quantitatively retained in the ether-butanol fraction.

(3) Hydrolysis of the hydrazone from the Girard derivative of estrone sulfate at pH 2.0 and room temperature for 6 hours was about 85–90% complete and less than 10% of the estrone sulfate was thus hydrolyzed. This freed estrone was readily removed by extraction with ether.

(4) and (5) The estrone sulfate was then separated out into a butanol-ether solution (Fraction 6).

This modified Girard procedure yielded over 60% of the sodium estrone sulfate (NaES) started with at step (1) (see Table 1). It was felt that this was adequate for trial application to urines.

*Application of the Modified Girard Procedure to Human Pregnancy Urine Concentrates.* The conjugated estrogens were removed from urine by acidification of the urine to pH 3 (Cohen, 1950a) and extracting twice with $\frac{1}{2}$ volumes of butanol for 8 hours each time in the continuous liquid-liquid extraction apparatus described by Cohen (1950b). The combined butanol

extracts were adjusted to approximately pH 7 with NaOH and concentrated *in vacuo.*

In order to aid the solution of the urinary butanol residues in the pyridine-acetic acid mixture prior to carrying out the Girard reaction about 20% of water was also added. This water was found to have no significant effect on the formation of the Girard complex from estrone sulfate.

The modified Girard Procedure (see Diagram 1) was applied to the butanol residues obtained from 1 liter batches of urine; in some cases known amounts of estrone sulfate were first added to the urine samples before extraction with butanol. The final butanol-ether solutions (Fraction 6, Diagram 1) were evaporated to dryness, the residues dissolved in water, acid-hydrolyzed, fractionated and assayed by the procedures referred to under Methods. The results of one group of typical experiments are shown in Table 1. About 10–15% of the total "estrone" conjugate of human pregnancy urine (as assayed after acid hydrolysis and fractionation) appeared in the concentrate obtained by subjecting the urines to the modified Girard procedure. However, since a similar portion of estriol conjugate was also thus concentrated, this concentration might well have been due to the solvent partitioning of glucuronide conjugates rather than to a concentration of urinary estrone sulfate. An average of 44% (30–60% for 5 urines) of the sodium estrone sulfate added to the BuOH residues obtained from either normal male urine or from pregnancy urine appeared in the final BuOH-ether fractions resulting from the application of the modified Girard procedure to these residues. The relatively low recoveries of the NaES added to urines as compared to that of NaES in water may be more apparent than real because of the destruction and losses of the estrone occurring during the hydrolysis and fractionation procedures necessary in the assay of the estrogen in urines. These data together with that reported in the following sections made it appear that no more than a small portion of the conjugated estrone in human pregancy urine could be estrone sulfate. It thus seemed that further experiments designed to enable a more quantitative concentration of any estrone sulfate in human pregnancy urine were inadvisable at this time.

B. *Partition Solubilities of the "Estrone" Conjugate of Human Pregnancy Urine and of Pure Estrone Sulfate*

During the course of the experiments outlined in the previous paragraph as well as in others which were devised to concentrate the estrone conjugate of human pregnancy urine, it appeared that the partition of the urinary conjugate between butanol and aqueous solutions differed from that of sodium estrone sulfate. This was tested as follows: Three samples of estrone sulfate, each containing 0.93 mg. estrone were dissolved in three

50 cc. lots of butanol and these were extracted 5 times with 0.2 volumes of (a) water, (b) 10% NaCl and (c) N. NaOH respectively. After adjusting the alkaline solutions to about pH 7, aliquots were evaporated to dryness under a stream of nitrogen and assayed directly. Three 50 cc. samples of the butanol concentrate (adjusted to approximately pH 7) of pregnancy urine (Batch LPU 208) (each equivalent to 500 cc. urine and containing 0.45 mg. of estrogen which appeared in the "estrone" fraction after acid hydrolysis) and 3 to which sodium estrone sulfate (equivalent to 0.38 mg. of estrone) was first added were subjected to similar extractions with

TABLE 2. DISTRIBUTION OF SODIUM ESTRONE SULFATE (NaES) AND OF URINARY "ESTRONE" CONJUGATE BETWEEN BUTANOL AND AQUEOUS SOLUTIONS

| | "Estrone" in partition solvents | | |
|---|---|---|---|
| | NaES | Pregnancy urine (500 cc. L.P.U. 208) | Pregnancy urine (500 cc. L.P.U. 208) + NaES (containing 0.38 mg. estrone) |
| | mg. | mg. | mg. |
| Original "Estrone" Present | 0.93 | 0.45 | 0.83 |
| Partition (a) BuOH | 0.54 | 0.10 | 0.28 |
| $H_2O$ | 0.35 | 0.24 | 0.24 |
| Partition (b) BuOH | 0.85 | 0.22 | 0.46 |
| 10% NaCl | 0.02 | 0.20 | 0.15 |
| Partition (c) BuOH | 0.84 | 0.15 | 0.41 |
| N. NaOH | 0.01 | 0.23 | 0.22 |

aqueous solutions. The alkaline solutions were brought to pH 7, the butanol solutions were evaporated to dryness *in vacuo*, the residues each dissolved in 50 cc. water, and these together with the aqueous extracts were hydrolyzed with acid, fractionated and assayed. The data obtained are summarized in Table 2. Confirmatory data were obtained in two other similar experiments.

It is obvious that the urinary estrone conjugate has a greater solubility in the aqueous phases than does sodium estrone sulfate. Losses in "estrone" sustained during the distribution and assay procedures are probably responsible, in large part at least, for the relatively low total "estrone" recoveries for the urines (an average of 68% for the 3 urines thus studied) and of the recovery of the NaES added to the urines (an average of 62% for the 3 urines). The data obtained in these experiments indicate that the presence of urine substances extracted by butanol have no significant effect on the distribution of sodium estrone sulfate between butanol and the aqueous solutions, since most, if not all, of the added NaES appeared in the butanol fractions.

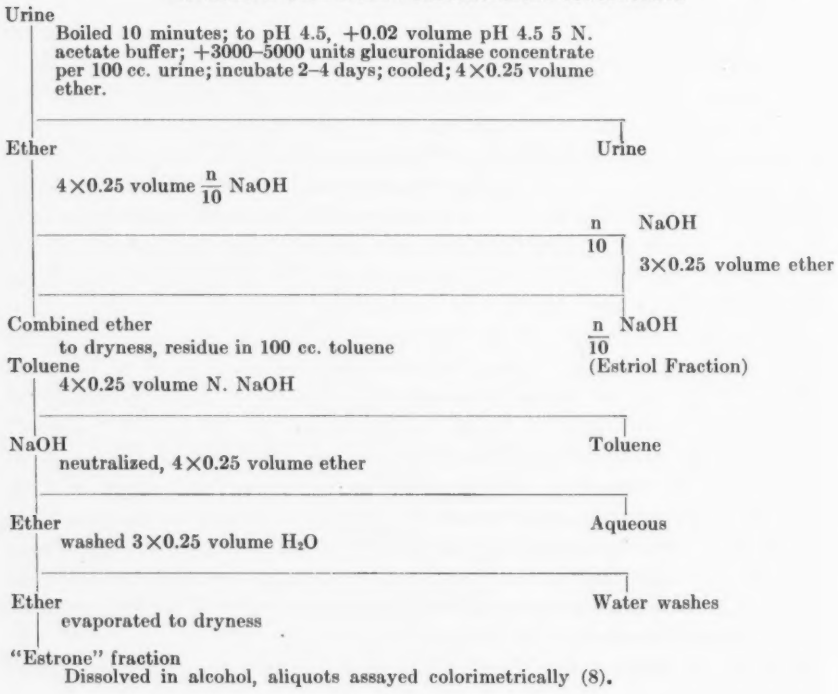
It is also of interest to note that the partition of the urinary "estrone"

fraction paralleled that of the estriol fraction in the butanol-water and butanol-salt solution distributions (also see Cohen, 1950a). This observation is not inconsistent with the concept that a major portion of the estrone, like estriol is present in human pregnancy urine as the glucuronide. While only insignificant amounts of NaES were removed from butanol solutions by N. NaOH, 64% (59–70%) of the recovered "estrone" conjugate and 88% (85–92%) of the recovered estriol of human pregnancy urine was thus removed. The greater proportion of the urinary estriol conjugate in the alkali than of the "estrone" conjugate may be explained by the influence of the free phenolic group of estriol glucuronide on the solubility of this compound in alkaline solutions (Cohen, 1950a). Estrone is probably conjugated through its phenolic hydroxyl group, thus making it unavailable for additional salt formation.

C. *Hydrolysis of Estrone Conjugate of Human Pregnancy Urine with Calf Spleen Glucuronidase Concentrates*

The possibility that the estrone glucuronide is the major form of con-

DIAGRAM 2. FRACTIONATION OF ESTROGENS IN HUMAN PREGNANCY URINE AFTER HYDROLYSIS WITH SPLEEN GLUCURONIDASE CONCENTRATE



jugated estrone in human pregnancy urine was tested by subjecting pregnancy urine specimens to hydrolysis with preparations of β-glucuronidase (Cohen, 1951) a procedure which has been found to have no hydrolytic effects on NaES dissolved in either water or urine. An outline of the procedures for glucuronidase hydrolysis and subsequent extraction for assay of the urines is shown in Diagram 2. The "estrone" fractions released by acid hydrolysis of separate aliquots of the urines were also determined. The data obtained are summarized in Table 3. An average of 91% (58–112%) of the acid released "estrone" was also released by the β-glucuronidase preparations. Control samples of urine similarly prepared and incubated without the spleen glucuronidase showed no detectable "estrone"

TABLE 3. COMPARISON OF "ESTRONE" RELEASED FROM HUMAN PREGNANCY URINE BY ACID AND BY ENZYME

| Urine Sample No. | "Estrone" liberated by hydrolysis with | | $\frac{(b)}{(a)} \times 100$ |
|---|---|---|---|
| | (a) HCl | (b) glucuronidase | |
| | mg./l. urine | mg./l. urine | |
| 1. | 0.7 | 0.5 | 71 |
| 2. | 1.5 | 1.6 | 107 |
| 3. | 1.2 | 0.7 | 58 |
| 4. | 1.1 | 0.9 | 82 |
| 5. | 0.9 | 0.8 | 89 |
| 6. | 0.8 | 0.8 | 100 |
| 7. | 0.9 | 0.9 | 100 |
| 8. | 0.8 | 0.9 | 112 |
| 9. | 1.2 | 1.2 | 100 |

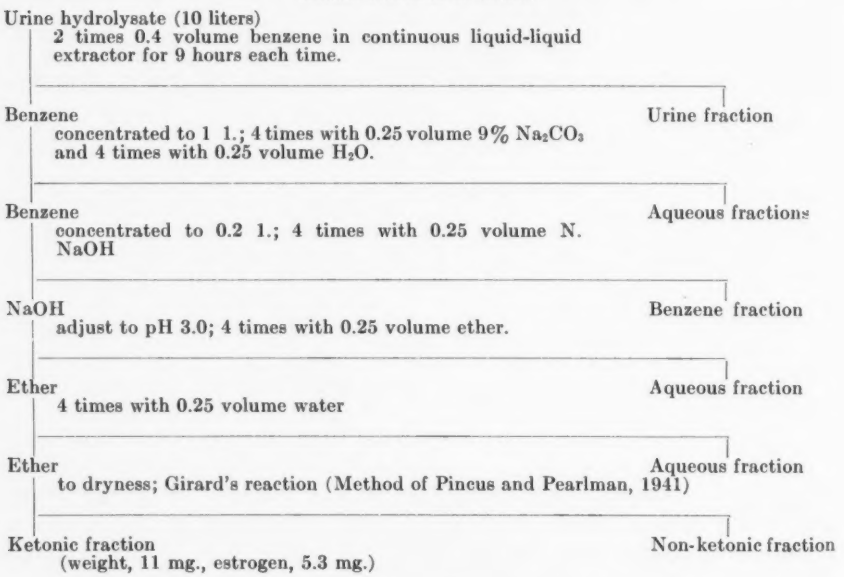
to have been released. The free (ether-soluble) "estrone" in the unhydrolyzed urine specimens was determined for all urines and in no case did it represent more than 1% of the "estrone" found after acid hydrolysis. Similarly low values for free estrogens in pregnancy urine have been previously reported by a number of workers (Cohen *et al.*, 1935; Browne *et al.*, Palmer, 1938; Dingemanse *et al.*, 1939; Hain, 1942; Clayton and Marrian, 1950).

*Isolation of Estrone from Glucuronidase Hydrolyzed Human Pregnancy Urine.* The presence of estrone as the major constituent of the estrone-estradiol fraction obtained by hydrolysis of the urines with calf spleen glucuronidase concentrates was established by isolation.

A 10 l. batch of human pregnancy urine, and containing 8 mg. of estrogen in the "estrone" fraction (see urine sample No. 6, Table 3) was boiled, buffered and incubated with 360,000 units of the glucuronidase preparation for 4 days. After cooling, the hydrolysate was extracted twice with 4 l. of benzene each time in the continuous extractor (Cohen, 1950b). Benzene was used as the extraction solvent rather than the highly volatile ether in order to permit the use of the continuous extraction apparatus for this

large volume of urine. The benzene extract was fractionated by the procedure of Bachman and Pettit (1941) with slight modifications. The fractionating procedure is shown schematically in Diagram 3. A ketonic fraction containing 5.2 mg. of estrogen (assayed colorimetrically) in 11 mg. of total residue was obtained. This fraction was not readily crystallized and was therefore sublimed at 100° and 5 microns pressure, yielding six

DIAGRAM 3. THE ISOLATION OF ESTRONE FROM A BETA-GLUCURONIDASE HYDROLYSATE OF HUMAN PREGNANCY URINE


Urine hydrolysate (10 liters)
2 times 0.4 volume benzene in continuous liquid-liquid extractor for 9 hours each time.
Benzene
Urine fraction
concentrated to 1 l.; 4 times with 0.25 volume 9% $Na_2CO_3$ and 4 times with 0.25 volume $H_2O$.
Benzene
Aqueous fractions
concentrated to 0.2 l.; 4 times with 0.25 volume N. NaOH
NaOH
Benzene fraction
adjust to pH 3.0; 4 times with 0.25 volume ether.
Ether
Aqueous fraction
4 times with 0.25 volume water
Ether
Aqueous fraction
to dryness; Girard's reaction (Method of Pincus and Pearlman, 1941)
Ketonic fraction
(weight, 11 mg., estrogen, 5.3 mg.)
Non-ketonic fraction

mg. of sublimate (m.p. 217–219°[3]). On crystallization from aqueous ethanol 2.2 mg. (m.p. 249–251°) of crystals which assayed quantitatively as estrone, were obtained. These showed no melthing point depression when mixed with an authentic sample of estrone (m.p. 249–251°). Further identification was prevented by the scarcity of material. However, these tests strongly indicate that the isolated ketonic estrogen is estrone.

## DISCUSSION

Final proof of the nature of the estrone conjugate or conjugates in human pregnancy urine must await isolation experiments. Such an isolation, however, represents a difficult problem in view of the relatively low

[3] The melting point determinations were carried out on a Fisher micro melting point apparatus and are uncorrected.

concentration of estrone in such urine (0.5–1.5 mg./liter of late pregnancy urine).

The hydrolysis of a large part of this estrone conjugate, by preparations of spleen glucuronidase is, however, strong positive evidence that this conjugate is a glucuronide. This is particularly so in view of the fact that the glucuronidase hydrolysis technique employed effects hydrolysis of steroid glucuronides but not of steroid sulfates (Cohen, 1951)

Although Butenandt and Hofstetter (1939) claimed that the estrone conjugate of human pregnancy urine resembled estrone sulfate in its properties, no experimental details were given. Their paper contained only a brief comparison of the properties of the respective conjugates such as solubility in butanol, insolubility in ether, precipitation with alkaloids, etc. These properties might equally well belong to estrone glucuronide.

Cohen and Bates (1949) have reported the hydrolysis of 5 to 89 per cent of the estriol and of 8 to 100 per cent of the estrone-estradiol conjugates of human pregnancy urine by Mylase P, a phenolsulfatase preparation. An inspection of their data, however, reveals that the values in the higher ranges were obtained from urines for which no controls (incubation of the urines without Mylase P) were run or from butanol extracts which contained only a fraction of the total estrogen content to be expected from the urine equivalents. Data on the poor removal of estriol glucuronide from near neutral aqueous solutions by butanol appeared (Cohen, 1950) some time after their work was completed. Losses of at least 50% of the estrone conjugate in human pregnancy urine to the aqueous phase in a butanol-water parition is indicated by the data presented in this paper (see Table 2). Hydrolytic experiments with Mylase P on 2 urine specimens by the technique of Cohen and Bates carried out in our laboratory resulted in the hydrolysis of about 20% of the estrogen conjugates in the urines. Similar treatment of urines first sterilized by boiling resulted in no more than a 5% hydrolysis, indicating that a considerable hydrolysis of the estrogen conjugates may be effected by glucuronidase already present in the urine. It is also possible that the Mylase P preparations contain small amounts of glucuronidase not measurable by the Fishman procedure (1948), which involves conditions of pH and temperature quite different from those employed for the Mylase P hydrolysis. It should be emphasized, however, that the data presented in this paper do not exclude the possible existence of some estrone sulfate in human pregnancy urine.

## SUMMARY

1. The Girard procedure for the separation of ketonic from non-ketonic steroids was modified so as to permit the concentration of estrone sulfate.

2. Application of this procedure to human pregnancy urines effected no appreciable concentration of the estrone conjugate.

3. The partition solubilities of the urinary conjugate differ from those of estrone sulfate.

4. Most of the "estrone" conjugate in pregnancy urine is hydrolyzed by preparations of spleen β-glucuronidase.

5. A ketonic faction containing 5.3 mg. of estrogen was isolated from 10 liters of pregnancy urine. This yielded 2.2 mg. of crystalline material which was apparently identical with estrone.

6. These data are interpreted as indicating that a large proportion of the estrone in human pregnancy urine is conjugated as the glucuronide.

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# HORMONAL INTERACTION IN THE REGULATION OF GRANULATION TISSUE FORMATION

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IN OUR previous investigations (Taubenhaus and Amromin 1949–1950) it was shown, that estradiol, testosterone, ACTH and cortisone exhibit a suppressing effect upon the granulation tissue which forms around turpentine abscesses in rats. Desoxycorticosterone (DOCA) and anterior pituitary growth hormone on the other hand promote formation of such tissue. Thyroxin stimulates granulation tissue only in thyroidectomized or hypophysectomized animals. The present study is an attempt to demonstrate synergistic or antagonistic effects, following the treatment of the same animal with two different hormonal substances. Methylandrostenediol was included in these studies, because this material was shown to have some of the metabolic properties of testosterone, but to produce minor changes in secondary sex characters (Henderson and Weissberg, 1951). Cortisone was chosen as the basic substance, the antagonistic and synergistic actions of the other hormones being tested in relation to its powerful inhibitory effect.

We utilized the unique property of the $C_{11}$ oxysteroids, which have been shown to inhibit granulation tissue locally, without exerting measurable effect upon areas distant from the site of administration (Baker and Whitaker, 1950; Castor and Baker, 1950; Meier et al., 1950; Taylor and Taubenhaus, 1951). In our previous experiments it was shown, that cortisone, mixed with turpentine and placed into one subcutaneous area inhibited granulation tissue at the site, and had no effect upon granulation tissue developing simultaneously around a turpentine abscess on the contralateral side

## METHODS

The general methods were the same as described in our previous publications. Male white rats of the Sprague-Dawley strain, weighing approximately 160 gms. were used in

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all experiments. In most instances 9 animals were used in each group. Turpentine U.S.P. (½ ccm.) was placed subcutaneously in the scapular region on both sides. On the fourth, fifth, and sixth day after induction of the abscesses three animals were sacrificed on each day. The abscesses were removed, fixed in formalin and examined, after staining with hematoxylin and eosin. No special fixation methods or stains were applied in these experiments, because our main interest was focused upon quantitative changes and the appearance of fibroblasts, which could well be studied with this method. The type and mode of administration of the various hormones used is included in the description of the individual experiments.

## RESULTS

The first series of experiments was designed to compare the inhibitory effect of Estradiol to that of cortisone. Four groups of animals were injected with turpentine on the left side. Estradiol propionate in doses of 0.001 mg., 0.01, mg., 0.1 mg. or 1 mg. was mixed with turpentine and injected on the right side, in each group respectively. On the fourth day after induction of the abscesses, the granulation tissue appeared definitely inhibited bilaterally. The inhibition was most pronounced in the group which received the higher doses of estradiol, although some effect was noted after injection of 0.01 mgm. Most of the animals which received 0.001 mg. produced granulation tissue, which did not differ from the normal controls. In no instance was a local (one-sided) action of the estrogen observed, indicating that the effect is systemic and is due to the estrogen absorbed from the turpentine mixture.

In order to test the influence of methylandrostenediol, 10 mg. of this substance was injected subcutaneously one day before the induction, and daily during the development of the turpentine abscesses. The granulation tissue of these animals showed a moderate, but definite inhibition, as compared with the controls. The same effect was observed in animals, in which the same amount of methylandrostenediol was placed mixed with the turpentine. An outspoken inhibition of granulation tissue was seen around this abscess, as well as on the contralateral side, into which turpentine alone was injected; indicating that the action of this steroid was a systemic one and lacked the characteristic local action of cortisone. The degree of inhibition was approximately the same, as observed previously with testosterone (Taubenhaus and Amromin, 1949). In another series of animals 10 mg. of methylandrostenediol was mixed with 1 mg. of cortisone and injected together with the turpentine over the right scapula of the animal. Turpentine alone was injected into the contralateral side. A marked inhibition of granulation tissue was found on the side containing the steroids, resembling the inhibiton by cortisone and a moderate inhibition resulted on the other side containing the turpentine alone, such as was seen in the methylandrostenediol experiments described above. No additive

effect was observed from the mixture of both steroids. The action of cortisone predominated locally. Methylandrostenediol, however, seemed to exert its effect systemically.

It appeared then of value to investigate whether hormones, which exhibited a stimulating effect upon granulation tissue would be able to counteract the cortisone effect. Thyroxin, desoxycorticosterone and growth hormone were examined in this respect.

One mgm. of thyroxin was injected hypodermically to intact animals starting one day before the turpentine abscesses were induced and continuing daily thereafter. The granulation tissue of such animals appeared normal and showed no stimulation. In another series of animals thyroxin was administered in the above manner to animals which were injected with plain turpentine on the left side and turpentine mixed with cortisone on the right side. The local, inhibitory effect of cortisone was quite pronounced on the right side. Thyroxin did not counteract the cortisone effect.

In our previous experiments desoxycorticosterone had a stimulating effect upon granulation tissue only, if the animals were pretreated with this substance for several weeks prior to the induction of abscesses. No such effect could be observed if DOCA was injected only during the development of granulation tissue. In order to test a possible local effect of this substance, 1 mgm. of DOCA mixed with turpentine was injected into the right side and turpentine alone was placed on the left. As expected, no effect upon the quantity or quality of granulation tissue was noted on either side. In another series of animals, which has received daily injections of DOCA for two weeks prior to the induction of abscesses, turpentine mixed with 1 mgm. of cortisone was placed on the right side and turpentine alone on the left side of the animal. DOCA injections were then continued during the period of abscess development. In control series without DOCA the cortisone inhibited granulation tissue strongly at the site of its injection (Fig. 1L and 1R). In the animals which had received DOCA inhibitory effects of cortisone were counteracted. Considerable amounts of granulation tissue could be seen on both sides. (Fig. 2L and 2R) The quality of this granulation tissue, on both sides, however, differed from the normal. It appeared less mature, the fibroblasts lacking the regular orderly arrangement parallel to the abscess cavity, and differing from the spindle shaped normal fibroblasts (Fig. 5A and B). On the whole it appeared similar to the granulation tissue developing under DOCA influence as observed by Taubenhaus and Amromin (1949), although the fibroblasts were not as large and stellate and the amount was not as great. The difference may be explained by the fact, that in our previous experiments, the animals were pretreated for a longer period of time (five weeks).

The influence of the anterior pituitary growth hormone* on the inhibitory function of cortisone was studied in rats, which had been hypophysectomized 5 days prior to the induction of turpentine abscesses. In previous experiments it was shown that such animals are particularly suitable for the study of growth hormone action upon granulation tissue.

In the first series of hypophysectomized animals which did not receive growth hormone injections, there was a failure of granulation tissue to develop on both sides. The inhibitory effect of cortisone could however still be observed. The granulation tissue on the left side, surrounding the turpentine abscess was sparse and thin. On the right side, which contained the turpentine mixed with 1 mg. of cortisone areas were seen, where no granulation tissue formed at all (Fig. 3L and 3R). Growth hormone was injected into three series of similarly treated animals. The injections were begun one day prior to the administration of the turpentine and cortisone respectively, and was continued daily, while the abscess developed. The first of these series received 5 gamma of growth hormone. The effect was small, as compared with the controls. Only one animal seemed to develop granulation tissue on both sides, which exceeded that of the controls. The second series received 50 gamma of growth hormone. Four of these animals showed granulation tissue development on both sides. The inhibiting effect of cortisone was either not apparent, or very insignificant (Fig. 4L and

*Armour preparation, lot K-40805R contaminated to the extent of approximately 23% in terms of the TSH standard 2R3.

FIGS. 1–4 inclusive (× 100). Illustrating granulation tissue in walls of five day old turpentine abscesses. Zone of necrosis and polymorphonuclear leucocytes on extreme right of each figure. Adjacent to it on the left is the granulation tissue layer.

FIG. 1L. Abscess developing around plain turpentine in the left scapular region.

FIG. 1R. Abscess around cortisone-turpentine suspension on the right side of same animal. Note scarcity of granulation tissue.

FIG. 2L. Animal treated with desoxycorticosterone. Left scapular region (turpentine alone).

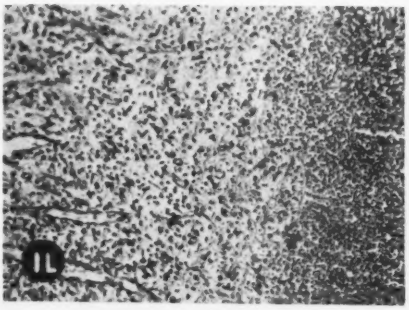
FIG. 2R. Same animal. Right scapular region (cortisone-turpentine suspension). Note that amount of granulation tissue has been restored to normal.

FIG. 3L. Hypophysectomized animal. Left scapular region (turpentine alone). Note scarcity of granulation tissue as compared to Fig. 1L.

FIG. 3R. Right side of same animal. Turpentine-cortisone suspension. Note that granulation tissue is nearly absent.

FIG. 4L. Hypophysectomized animal treated with growth hormone. Left scapular region containing turpentine alone. Note that ample granulation tissue has developed as compared to Fig. 3L.

FIG. 4R. Right side of same animal containing cortisone-turpentine suspension. Note that no inhibition is noted and the granulation tissue has been restored to normal as compared to Fig. 3R.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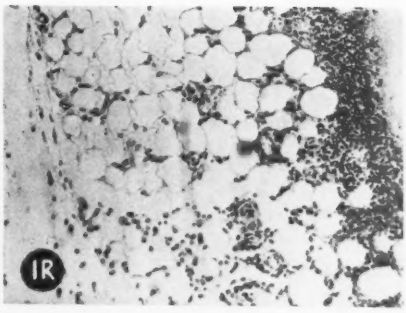
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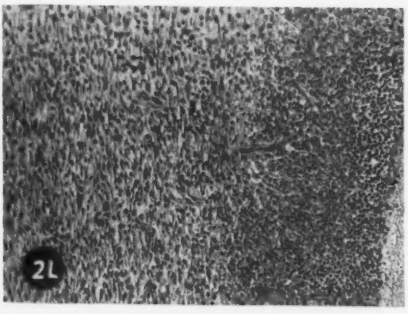
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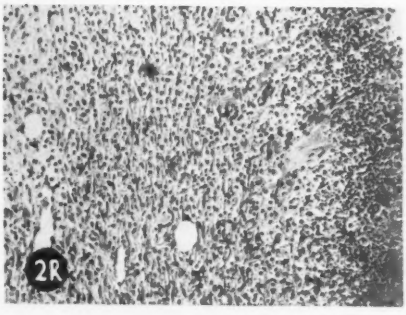
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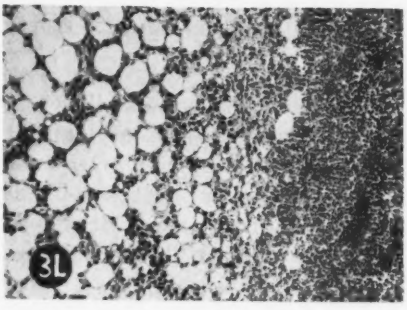
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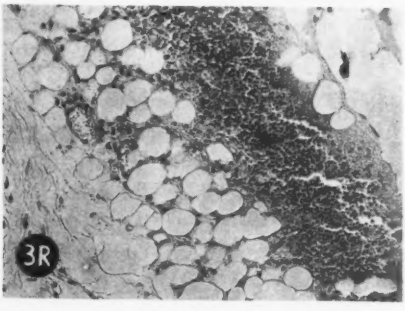
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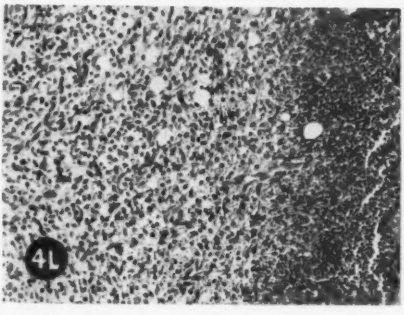
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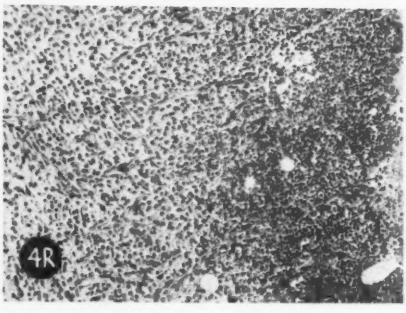
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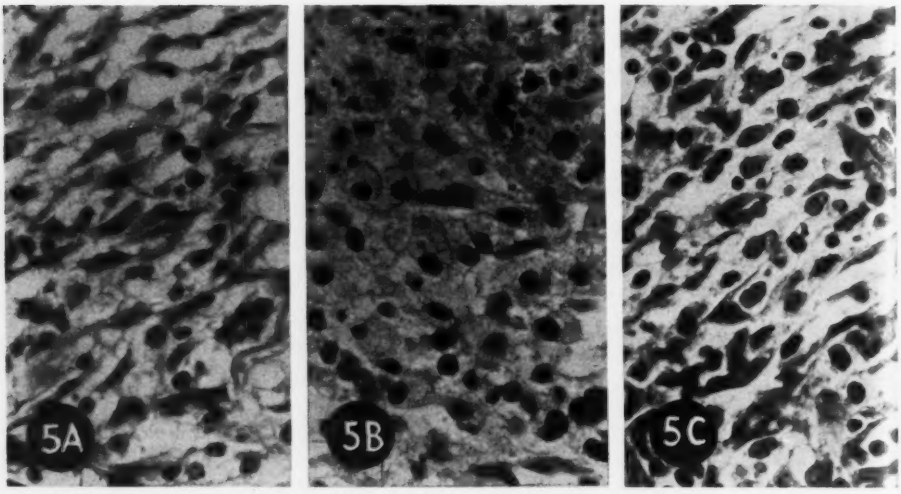


FIG. 5A. Granulation tissue surrounding turpentine abscess, ×600. Note spindle shaped fibroblasts, arranged in parallel fashion.

FIG. 5B. Animal treated with desoxycorticosterone. Granulation tissue of turpentine cortisone suspension. Higher magnification (× 600) of 2R. Note that tissue appears less mature and no parallel arrangement of fibroblasts can be seen.

FIG. 5C. Hypophysectomized animal treated with growth hormone. Granulation tissue surrounding cortisone-turpentine suspension. Higher magnification (× 600) of Fig. 4R. Note similarity to normal control animal.

4R). The third series received 500 gamma of growth hormone. The majority of these animals showed excellent granulation tissue development on both sides and the suppression by cortisone was completely overcome, except in one animal. The quality of the granulation tissue in these animals was normal in contradistinction to the granulation tissue which developed under the action of DOCA, as described above. The fibroblasts were spindle shaped and arranged in orderly fashion parallel to the abscess cavity and the whole tissue gave the impression of a good general organization, such as seen in normal animals (Fig. 5C).

## DISCUSSION

Table 1 summarizes schematically the results of our experiments. The inhibitory effect of estradiol upon granulation tissue formation previously described by Taubenhaus and Amromin (1949) was corroborated by Portugal and associates (1951) and by Lichtwitz and co-workers (1951). It does not require pretreatment of the animals with this hormone as believed by Clayton and Prunty (1951). This is well demonstrated in our present experiments, in which the estrogen was mixed with the turpentine and acted by absorption from the abscess cavity. As soon as the inhibitory

influence of estradiol became apparent, both sides of the animal were affected alike, indicating, that this effect is a systemic one as suggested before, most likely through the inhibition of the production or release of growth hormone by the anterior pituitary. A local effect, such as exerted by cortisone, could never be observed in these animals.

Methylandrostenediol, which has metabolic effects similar to testosterone, but less pronounced androgenic manifestations (Henderson and Weinberg, 1950), has a moderately inhibitory action upon granulation

TABLE 1

| Type of experiment: | Amount of granulation tissue. (3: well developed 2: moderate 1: sparse) |
|---|---|
| 1. right: turpentine alone | 3 |
| left: turpentine alone | 3 |
| 2. right: turpentine with cortisone | 0–1 |
| left: turpentine alone | 3 |
| 3. right: turpentine with estradiol | 1–2 |
| left: turpentine alone | 1–2 |
| 4. right: turpentine with methylandrostenediol | 2 |
| left: turpentine alone | 2 |
| 5. right: turpentine with cortisone | 3 |
| left: turpentine alone | 3 |
| systemically: desoxycorticosterone | |
| 6. right: turpentine with cortisone | 0–1 |
| left: turpentine alone | 1 |
| (hypophysectomized animals) | |
| 7. right: turpentine with cortisone | 3 |
| left: turpentine alone | 3 |
| systemically: growth hormone | |
| (hypophysectomized animals) | |

tissue, resembling testosterone. Again the effect is a systemic one, like the one observed with estrogen, although much less pronounced. It is interesting to note that the high protein anabolic activity of methylandrostenediol did not lead to an excess of granulation tissue formation and the inhibitory effect must have a more specific basis. It must be remembered though that these experiments do not permit any conclusions in respect to actual wound healing, which may very well be enhanced by this substance.

Thyroxin, which proved to have a stimulatory effect in hypophysectomized or thyroidectomized animals, has no discernible effect upon granulation tissue of normal rats and no stimulating influence upon the cortisone inhibited tissue.

Desoxycorticosterone failed to exert an effect upon granulation tissue if administered only while such tissue is in process of development. Pretreatment of the experimental animals was necessary to obtain the stimulating effect described by us and corroborated by Pirani and associates (1951). It was observed though that this excessive granulation tissue shows qualitative differences as compared with granulation tissue of

normal animals. Pirani and associates (1951) also pointed out that in spite of exuberant granulation tissue, wound healing as such is delayed, probably because an abnormal type of granulation tissue is formed. The mechanism of action of desoxycorticosterone upon granulation tissue and the reason why no immediate effect can be observed has not been explained. It is possible that the effect is an unspecific one and is due to electrolyte shifts which take place before the turpentine has been injected. The fact that cortisone has an entirely different effect upon granulation tissue, although the influence upon the electrolyte pattern may be similar is somewhat against such an assumption. Cortisone inhibition of granulation tissue can be counteracted by desoxycorticosterone as shown in our experiments. However, this antagonism refers only to the quantity of granulation tissue, its quality being abnormal, as discussed before. It might be stressed here again that the tissue on the cortisone injected side as well as in the contralateral abscess appeared abundant, but less well organized, less mature and differences were observed in the appearance of the fibroblasts. DOCA is therefore not a true antagonist of cortisone. On the other hand some of the vascular changes induced by DOCA can be counteracted by cortisone. This has been described before and the pertinent literature reviewed by Selye (1951).

The stimulating effect of growth hormone, which was described previously (Taubenhaus and Amromin, 1950) and recently elaborated and stressed by Selye (1951), is also noticeable in granulation tissue which has been under the inhibiting influence of cortisone. Observing such tissue, one gains the impression, that it has been restored to normal. The quality and the quantity does not differ from the control side. Compared with granulation tissue, which has been stimulated by DOCA a striking difference can be noted. Although both these hormones exhibit a stimulating effect, the mechanism must be different, because of the different appearance of the granulation tissue under the influence of either one of these hormones. It might be stressed here again, that DOCA has an indirect effect in that it does not work immediately, while the granulation tissue is developing. On the contrary, growth hormone has an immediate and direct effect, as shown in our experiments. The assumption could be made that cortisone inhibits granulation tissue by counteracting the effect of growth hormone upon the target organ and that both hormones operate upon the factors responsible for fibroblastic proliferation

## SUMMARY

Hormonal influences upon granulation tissue forming around turpentine abscesses of white rats were studied. In previous experiments it had been shown, that cortisone suspended in turpentine and injected subcutaneously exerted a local inhibitory effect and did not alter granulation tissue

developing on the contralateral side of the same animal, where turpentine alone was injected.

Estradiol suspended in turpentine exerted an inhibitory effect also upon granulation tissue developing in the distant area, indicating that its effect is a systemic one, unlike cortisone.

Methylandrostenediol caused a moderate inhibition of granulation tissue, resembling the action of testosterone. Suspended in turpentine, it inhibited such tissue also in distant areas, to a lesser degree than estradiol.

Thyroxin had no effect on granulation tissue in intact animals and did not interfere with the cortisone inhibition.

Desoxycorticosterone injected hypodermically counteracted the local inhibitory effect by stimulating granulation tissue at the site of the cortisone-turpentine suspension. However it led the development of a less organized, immature, "abnormal" granulation tissue.

The local inhibitory effect of cortisone could be still observed in hypophysectomized animals, in which granulation tissue formation is generally diminished.

Anterior pituitary growth hormone administered to such animals completely counteracted the inhibition by cortisone and caused ample granulation tissue formation around turpentine abscesses with and without cortisone. The finer structure of such granulation tissue showed normal organization and morphology. It was different from the type of tissue developing under the influence of desoxycorticosterone. The significance of these findings was discussed.

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# INHIBITION OF PHOSPHORYLASE WITH CORTISONE AND ITS ACTIVATION WITH ADRENALINE IN THE RABBIT

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SINCE Browne, in 1943, demonstrated that adrenocorticotropic hormone or ACTH reduces glucose tolerance and causes glycosuria in connection with glucose tolerance test in man, a comprehensive literature on the subject has appeared. Long (1947) was the first to state that ACTH effect arises through stimulation of the adrenal cortex which releases steroids active in carbohydrate metabolism. Of these steroids, or glyco-corticoids, 11-dehydro-17-hydroxycorticosterone or compound E, also called cortisone, has become widely used as a therapeutic preparation. Ingle and associates (1945–46) first showed that prolonged glycosuria and hyperglycemia can be produced with cortisone. Alimentary, insulin-resistant diabetes, unaccompanied by acidosis, may also develop. There are a great number of reports dealing with this question too.

In adrenalectomized animals the glycogen content of the liver can be restored with cortisone, and even overcompensated (Olson et al. 1944, Venning et al. 1946). Teilum and associates (1950) produced intense glycogen deposition in the liver and kidneys of rabbits with large doses of cortisone.

It has long been known that the adrenal medullary hormone, i.e. adrenaline or epinephrine, is capable of mobilizing sugar rapidly from glycogen stores. The blood sugar rises, glycolysis is accelerated and its end-product, lactic acid, increases in the blood.

Though the effect of ACTH and cortisone on the carbohydrate metabolism has been extensively studied, its mode of action has proved very difficult to explain. The theory most generally accepted is that sugar is formed from proteins and possibly from fat, in other words that glyconeogenesis is stimulated. This gives rise to a source of energy, easy to mobilize (Albright 1943). The sugar produced in excess is then deposited as glycogen or released by the kidneys. This theory is mainly based on the observation that the substances concerned often cause a negative nitrogen balance. However, the phenomenon does not occur regularly and bears no direct correlation to the disturbance in carbohydrate metabolism. Individual

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variations in insulin production have also been suggested as an explanation. Ragan and co-workers (1949) believe the ACTH effect to consist in hyperadrenalism and Paulsen (1950) interprets it as polymerization of carbohydrates especially into mucopolysaccharides. Leupin and Verzár (1950) assume that there occurs a disturbance in the initial phosphorylation of sugar.

### THE EFFECT OF CORTISONE ON LIVER AND MUSCLE GLYCOGEN

The glycogen content of the liver was studied in 4 untreated rabbits and in 12 rabbits after intramuscular cortisone treatment (Cortone Merck & Co., Inc. Rahway, N. J., U.S.A.) extending over 7 days. Glycogen was determined by the method of Pflügler as modified by Haex (1944).

TABLE 1. LIVER AND MUSCLE GLYCOGEN IN UNTREATED ANIMALS (CASES 1–4) AND AFTER TREATMENT WITH CORTISONE FOR ONE WEEK (CASES 5–16)

| Cortisone mg. daily | Glycogen | |
|---|---|---|
| | In liver, per cent | In muscle, mg. per hundred grams |
| — | 0.35 | 646 |
| — | 0.32 | 573 |
| — | 0.59 | 142 |
| — | 0.64 | 195 |
| 1.25 | 2.80 | 540 |
| 2.50 | 1.30 | 722 |
| 4.00 | 1.20 | 230 |
| 4.00 | 2.50 | 130 |
| 5.00 | 8.40 | 595 |
| 6.00 | 8.80 | 400 |
| 10.00 | 13.50 | 2000 |
| 10.00 | 12.20 | 1100 |
| 15.00 | 14.50 | 2300 |
| 20.00 | 12.00 | 1700 |
| 20.00 | 11.00 | 1500 |
| 20.00 | 15.40 | 1000 |

In control animals the liver glycogen varied from 0. 32 to 0.64 per cent. Following cortisone administration the glycogen content rose progressively with increasing doses. Deposition of large amounts of glycogen in the liver was obtained with a daily dose of 5 mg. With larger doses the glycogen content increased further and reached 11–15 per cent following administration of 10–20 mg. daily for one week (table 1).

Muscle glycogen in untreated animals varied from 142 to 646 mg. per hundred grams. After cortisone treatment glycogen deposition in muscle was less than in the liver, with the same duration of treatment. But when 10–20 mg. of cortisone was administered for 7 days the glycogen content of muscle reached 1–2 per cent (table 1).

EFFECT OF CORTISONE ON PHOSPHORYLASE ACTIVITY

The last step in glycogen synthesis consists in the conversion of glucose-1-phosphate into glycogen, the reaction being glucose-1-phosphate $\underset{\longrightarrow}{\overset{\longleftarrow}{\text{phosphorylase}}}$ glycogen + inorganic phosphate. The enzyme phosphorylase appears in an active form *a* and in an inactive form *b*. Phosphorylase *b* can be converted into phosphorylase *a* by adding muscle adenylic acid. The *a* form, again, is converted into the *b* form through the action of a proteolytic ferment termed PR (Prosthetic group removing). These phenomena have been described by Green, Gerty Cori and Oncley (1943). A resting muscle contains mainly phosphorylase *a* and a strongly stimulated muscle mainly phosphorylase *b* (Gerty Cori 1945).

The phosphorylase is prepared and purified according to the method described by Green, Gerty Cori and Oncley for the preparation of crystalline phosphorylase.

The test animal, which has been allowed no food for the last 15 hours, is anesthetized with ether; the blood vessels of the neck are immediately sectioned and the animal sacrificed. The muscles of one hind leg are removed and the liver is dissected out. The muscles or liver are weighed and immediately taken into a cold room where the following steps are carried out. The muscles (liver) are passed twice through a meat grinder and extracted twice for 10 minutes, during mixing, with a volume of cold water corresponding to the weight of the muscles (liver). The extract is strained off through gauze and then through soft filter paper. The pH of the extract is adjusted with dilute HCl to 6.0–6.2. It is then dialyzed in a cellophane tube (diameter 24 mm.) against running water (5–10° C.) for 3 hours. The pH is brought to 5.8–5.9 with 0.03-n HCl. After a while an isoelectric precipitate forms containing the PR enzyme. The precipitate is rapidly removed by centrifugation and filtration. The first determination of phosphorylase activity is made from the clear filtrate (1st fraction).

The filtrate is then neutralized with about 1 Gm. sodium-$\beta$-glycerophosphate per 100 cc. of solution. 0.7 volume of ammonium sulfate solution saturated at room temperature is added to make the final solution 41 per cent saturated and of pH 6.8. A small quantity of precipitate settles overnight. The supernatant fluid is decanted and the rest collected by centrifugation. 25 to 33 per cent of (the protein in) this precipitate is phosphorylase. The precipitate is suspended in 10 cc. cold water and dialyzed against a glycerophosphate-cysteine buffer of pH 6.8 (1 cc. 0.3-M cysteine-HCl + 39 cc. 1 per cent sodium-$\beta$-glycerophosphate). The dialyzing fluid is changed a few times. The dialyzing bag is set in an ice bath. Phosphorylase *a* is precipitated overnight in crystalline form. If the phosphorylase *a* content is less than 25 per cent, no crystals will form. A second determination of enzyme activity is carried out (2nd fraction).

For activity measurements an aliquot of the enzyme solution is diluted 25 to 50 times with 0.03 M cysteine-HCl, whereupon the crystallized enzyme protein goes into solution. Determination of activity must be made 20-60 minutes after the dilution is prepared: immediately after it there may be a slight rise in activity (Cori, C. F., Gerty Cori and Green 1943).

The activity of phosphorylase *a* can be calculated from inorganic phosphate liberated from added glucose-1-phosphate. The composition of the reaction mixture is 0.016 M glucose-1-phosphate, 1 per cent glycogen, and 0.015 M cysteine 1 per cent glycerophos-

phate buffer, pH 6.8. Without addition of glycogen both enzyme forms are inactive. The pH optimum for the reaction is between 6.5 and 6.9. Cysteine glycerophosphate buffer depresses enzyme activity to some extent (Cori, C.F., Gerty Cori and Green). For determination of the total phosphorylase content the *b* form must be activated by preparing a reaction mixture with muscle adenylic acid $10^{-3}$ M.

Phosphorylase activity is measured as follows: To 1 cc. of 0.3 M cysteine-hydrochloride is added 39 cc. of 1 per cent sodium-$\beta$-glycerophosphate, and mixed. The pH of this solution is 6.8. 1 cc. of the solution is pipetted into four Erlenmeyer flasks with ground glass stopples. Two of the flasks are for activity determination, and two for control tests. 2 cc. of 2 per cent glycogen solution and 1 cc. 0.064 M neutralized glucose-1-phosphate, this latter containing no free inorganic phosphate, are added to all flasks, and further 1.4 mg. of muscle adenylic acid to two of the flasks. The reaction mixture is allowed to come to 30°C. in a bath. Now 0.2 cc. of the enzyme solution diluted with cysteine is added, mixed, and allowed to digest for 5 minutes. The reaction is then stopped by adding 4 cc. of 7 per cent trichloroacetic acid. The solution is mixed and allowed to cool. In the control tests the trichloroacetic acid is added immediately after addition of the enzyme solution. The reaction mixture is filtered and its content of inorganic phosphate is determined quickly in order that the acid should not have time to hydrolyze glucose-1-phosphate. Double determinations are made at all tests.

Inorganic phosphate is determined according to Bomskow's modification of Fiske-Subbarow's method (1932) in the Beckman spectrophotometer, Model "DU", at wavelength 740 m$\mu$. The blank reading is deducted from the result.

Glucose-1-phosphate is prepared according to Sumner and Somers (1944) and muscle adenylic acid according to Kerr (1941) and Needham (1942). The glycogen was manufactured by the firm Hoffmann—La Rôche, Basle, Switzerland.

The velocity constant (K) is used as a reference basis for the reaction rate equation. This constant is directly proportional to the activity (Cori, C. F., Gerty Cori and Green). The reaction is considered to be of the first order and the velocity constant then has the following equation:

$$K = \frac{1}{t} \log_{10} \frac{a}{a-x}$$

where $t$ = time in minutes, $a$ = quantity of reacting test substance, and $x$ = amount of test substance consumed. If the pH of the reaction mixture is adjusted to 6.8, as here, $a = 80$, which is the percentage of glucose-1-phosphate converted to polysaccharide (glycogen) at equilibium. In the equation, $x$ is then the amount of phosphorus liberated during the time $t$ in terms of percentage of total hydrolyzable phosphate.

The percentage of phosphorylase $a$ in the solution is obtained by dividing the velocity constant in the reaction mixture without addition of adenylic acid by the corresponding constant in the mixture with added muscle adenylic acid, and by multiplying this quotient by 100.

The results are seen in table 2, which shows the quantity of active phosphorylase *a*, in percentage of total phosphorylase, obtained by activation with muscle adenylic acid. The first fraction refers to the crude preparation of the enzyme, and the second to the purified preparation. The amount of phosphorylase present in liver is not sufficient to allow determination in the purified 2nd fraction. The percentage of phosphorylase *a* given in the

text corresponds to the average amount obtained in the 1st and 2nd fractions.

In 13 control animals phosphorylase *a* in muscle varied from 43 to 67 per cent; on an average its activity was 52 per cent. In liver (4 cases) the corresponding activity was 25–45 per cent, mean 34 per cent (table 2 and fig. 1).

Altogether 20 rabbits were treated with cortisone doses of 1.25 to 20

TABLE 2. PHOSPHORYLASE ACTIVITY (*a*) IN MUSCLE AND LIVER OF UNTREATED ANIMALS (CASES 1–13) AND AFTER CORTISONE TREATMENT FOR ONE WEEK (CASES 14–29)

| Cortisone mg. per day | 1st fraction | 2nd fraction | Average | In liver 1st fraction |
|---|---|---|---|---|
| — | 32 | 55 | 43.5 | — |
| — | 68 | 65 | 66.5 | — |
| — | 58 | 40 | 49 | — |
| — | 55 | 57 | 56 | — |
| — | 46 | 46 | 46 | — |
| — | 51 | 47 | 49 | — |
| — | 40 | 48 | 44 | — |
| — | 50 | 65 | 57.5 | — |
| — | 55 | 41 | 48 | — |
| — | 46 | 40 | 43 | 38 |
| — | 68 | 64 | 66 | 45 |
| — | 44 | 46 | 45 | 25 |
| — | 59 | 53 | 56 | 29 |
| 1.25 | 28 | 38 | 33 | — |
| 2.50 | 37 | 35 | 36 | — |
| 4.00 | 20 | 20 | 20 | — |
| 4.00 | 25 | 38 | 31.5 | — |
| 5.00 | 0 | 4.5 | 2.5 | — |
| 6.00 | 24 | 21 | 22.5 | — |
| 10.00 | 9 | 0 | 4.5 | — |
| 10.00 | 19 | 8 | 13.5 | — |
| 15.00 | 11 | 2 | 6.5 | 10 |
| 15.00 | 13 | 12 | 12.5 | 13 |
| 15.00 | 9 | 15 | 12 | 0 |
| 20.00 | 5 | 4 | 4.5 | — |
| 20.00 | 7 | 13 | 10 | — |
| 20.00 | 8 | 6 | 7 | 9 |
| 20.00 | 12 | 20 | 16 | 5 |
| 20.00 | 0 | 0 | 0 | 0 |

mg. daily. It was given by intramuscular injection twice daily, the last injection two hours before the animal was killed. The duration of treatment was one week. It appears from table 2 and figure 1 that the percentage of phosphorylase *a* decreased progressively with increasing doses of cortisone, to fall to extremely low values—even to nil—following treatment with very large doses. With a daily dose of 4–5 mg. cortisone for 7 days, phosphorylase activity fell to about one-half of the normal. In one exceptional case it was only 2 per cent, following administration of 5 mg. cortisone. A daily dose of 10–20 mg. cortisone depressed phosphorylase activity to about one-fifth. Occasionally the enzyme was wholly inactiv-

ated. The effect on liver phosphorylase was the same as on muscle phosphorylase. In some half-grown animals (fig. 1) phosphorylase activity seemed to fall less than with the same dose in adult animals: the average muscle phosphorylase content in 3 four-months-old rabbits treated with a daily dose of 15 mg. cortisone for one week was 20 per cent while the cor-

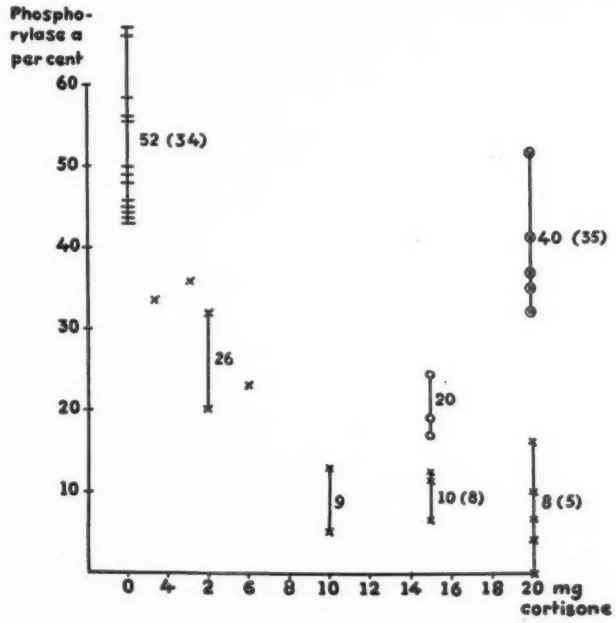


FIG. 1. Phosphorylase *a* in rabbit muscle before (I) and after (⚸) cortisone administration for one week, in half-grown animals after cortisone administration (⚷), and in cortisone-treated animals 15 minutes following intramuscular adrenaline injection (0.01 mg. per kg. body weight) (⚷). The figures indicate the average content of phosphorylase *a* in muscle and the figure in brackets the average content in liver.

responding average for 3 adult animals treated in the same way was 10 per cent.

Total phosphorylase activity with added muscle adenylic acid varied to some extent from animal to animal. However, it was always of the same order of magnitude and unaffected by the cortisone doses.

### EFFECT OF ADRENALINE ON PHOSPHORYLASE ACTIVITY AND ON BLOOD SUGAR BEFORE AND AFTER CORTISONE TREATMENT

Adrenaline (Parke, Davis & Co., London), 0.01 mg. per kg. of body weight, was injected into untreated and cortisone-treated rabbits. In

one series of experiments the animals were sacrificed 15 minutes following the injection and the phosphorylase activity in muscle and liver was then determined. In another series of tests the blood sugar was determined by Hagedorn-Jensen's method following an adrenaline injection before and after cortisone treatment.

In untreated animals adrenaline had no demonstrable effect on phosphorylase activity. In 5 rabbits treated initially with a daily dose of 20 mg. cortisone for one week, the amount of phosphorylase *a* present in muscle after adrenaline injection was slightly lower than in untreated

TABLE 3. PHOSPHORYLASE ACTIVITY IN CORTISONE TREATED ANIMALS (20 MG. DAILY FOR ONE WEEK) 15 MIN. AFTER ADRENALINE INJECTION (0.01 MG. PER KG. BODY WEIGHT)

| Per cent phosphorylase *a* | | | |
|---|---|---|---|
| In muscle | | In liver | |
| 1st fraction | 2nd fraction | Average | 1st fraction |
| 39 | 33 | 36 | 38 |
| 51 | 62 | 56.5 | 38 |
| 35 | 46 | 40.5 | 39 |
| 41 | 26 | 33.5 | 32 |
| 31 | 39 | 35 | 29 |

animals, while that in liver was equal (table 3 and fig. 1). The value obtained for cortisone-treated animals averaged 40 per cent in muscle and 35 per cent in liver, and that for untreated animals 52 per cent in muscle and 34 per cent in liver. In animals treated with similar doses of cortisone and given no adrenaline, the average content of phosphorylase *a* was 8 per cent in muscle and 5 per cent in liver.

As is known, adrenaline injection produces a rise in blood sugar in test animals. In the experiments a maximum as a rule occurred in untreated animals after half an hour; the blood sugar then decreased abruptly and returned to normal within 2–3 hours. In animals treated for four days with a daily dose of 10–20 mg. cortisone, the rise in blood sugar following upon adrenaline injection equalled that observed in untreated animals. But a maximum was generally reached half an hour later and the blood sugar values fell more slowly than in animals given adrenaline before cortisone treatment. This occurred regardless of whether or not the treatment produced permanent hyperglycemia in the animals. Blood sugar did not return to the original level within three hours (table 4 and fig. 2.)

## RESULTS AND DISCUSSION

The last step in glycogen synthesis, or the first in glycogen breakdown is catalyzed by the enzyme phosphorylase, the reaction being: glucose-1-

phosphate $\underset{\longleftarrow\!\!\!\!\!\!\longrightarrow}{\text{phosphorylase}}$ glycogen+inorganic phosphate. In the experiments phosphorylase activity was determined in the rabbit before and after cortisone treatment. Activity of phosphorylase is expressed in percentage of its activity with added muscle adenylic acid. In all cases the phosphorylase content was studied in muscle and in a number of cases also in liver.

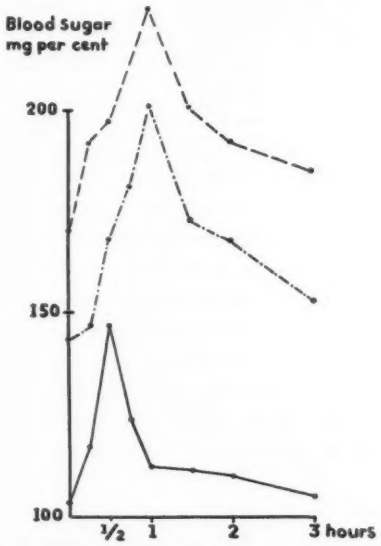


FIG. 2. Increase in blood sugar in rabbit following intramuscular injection of adrenaline (0.01 mg. per kg. of body weight) before (—) and after cortisone treatment (4 days), 10 mg. (·—·—·—·) and 20 mg. (— — — —) daily.

TABLE 4. INCREASE IN BLOOD SUGAR IN ANIMALS GIVEN ADRENALINE INTRAMUSCULARLY (0.01 MG. PER KG. BODY WEIGHT) BEFORE AND AFTER CORTISONE TREATMENT (4 DAYS)

| Blood sugar, mg. per hundred cc. of blood | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Before treatment | | | | | | | Cortisone, mg. | After treatment | | | | | | |
| min. | | | | | | | | min. | | | | | | |
| 0 | 15 | 30 | 60 | 90 | 120 | 180 | | 0 | 15 | 30 | 60 | 90 | 120 | 180 |
| 142 | 160 | 198 | 157 | 155 | 151 | — | 10 | 142 | 146 | 168 | 201 | 173 | 168 | — |
| 108 | 116 | 165 | 116 | 113 | 110 | 110 | 10 | 168 | 177 | 233 | 211 | 204 | 193 | 168 |
| 112 | 113 | 123 | 145 | 134 | 129 | 121 | 20 | 230 | 232 | 237 | 269 | 267 | 263 | 243 |
| 141 | 160 | 198 | 157 | 155 | 151 | — | 10 | 142 | 146 | 168 | 201 | 173 | 168 | — |
| — | — | — | — | — | — | — | 20 | 142 | 159 | 163 | 196 | 166 | 160 | — |
| 103 | 117 | 146 | 113 | 112 | 110 | 105 | 10 | 142 | 146 | 168 | 201 | 173 | 168 | 153 |
| — | — | — | — | — | — | — | 20 | 171 | 192 | 197 | 225 | 201 | 193 | 185 |

In untreated animals phosphorylase activity (*a*) in muscle varied from 43 to 67 per cent, with an average of 52 per cent. This agrees well with the figures reported by Gerty Cori (1945). Her corresponding values for 12 rabbits were in the 1st fraction 44 to 65 per cent, with an average of 60 per cent, and in the 2nd fraction 34 to 65 per cent, with an average of 54 per cent. In animals treated with cortisone for one week phosphorylase activity decreased progressively with increasing doses. Following administration of 4–5 mg. cortisone daily, enzyme activity fell to about one-half, and with daily doses of 10–20 mg. to about one-fifth. The enzyme can be rendered wholly inactive. Inactivation occurs on the whole parallelly in muscle and in liver. Half-grown test animals seemed to be more resistant to the inactivating action of cortisone than adult animals. Experiments with ACTH did not yield so distinct and uniform results as those obtained with cortisone. This seems to depend on the well-known fact that the effect of ACTH is transitory, which also causes difficulties in performing the tests. In some tests, however, ACTH administration produced distinct inhibition of phosphorylase activity.

The glycogen content of muscle and especially of liver increases in cortisone-treated animals; this has been demonstrated by previous observers. A daily dose of 5 mg. cortisone, for one week even, caused deposition of large amounts of glycogen in the liver. With a daily dose of 10–20 mg. the glycogen content of the liver increased by 20 to 30 times. Under similar conditions glycogen storage in muscles was less, yet considerable.

As described above, phosphorylase activity was calculated from inorganic phosphate liberated by addition of glucose-1-phosphate. This allows the conclusion that cortisone prevents glycogen formation in this last phase. However, the simultaneous great increase in the glycogen content of the tissues indicates that the breakdown of glycogen is at the same time even more inhibited. With the largest cortisone doses used in human therapy, phosphorylase activity can be maximally inhibited. At the same time the amount of glycogen present in tissues also reaches a maximum.

In the tests adrenaline injection was without effect on phosphorylase activity in untreated animals. In animals treated with large cortisone doses, which according to previous experiments reduces phosphorylase activity to very low values, this activity was found to be nearly normal following an injection of adrenaline. In such animals the rise in blood sugar remained unchanged after adrenaline administration, regardless of whether permanent hyperglycemia developed or not. But after cortisone treatment the maximum blood sugar value was as a rule observed half an hour later and the blood sugar returned more slowly to the original value. The re-

sults suggest that the enzyme inactivated by cortisone can be reactivated by means of adrenaline, though this process requires some time.

All the animal experiments seem to show that the organism has at its disposal a hormonally controlled mechanism regulating both glycogen storage and breakdown. The active enzyme is in both cases phosphorylase, as demonstrated by Cori and his group. Adrenaline stimulates glycogen breakdown by activating the phosphorylase. Cortisone (or ACTH) retards phosphorylase activity and renders storage of glycogen, and especially its breakdown, difficult. Adrenaline may produce the same effect in a later phase by stimulating pituitary activity. It seems that changes such as hyperglycemia, glycosuria, insulin-resistance and glycogen storage—which have been demonstrated in man and animals after treatment with large doses of cortisone (or ACTH) and which appear spontaneously in Cushing's syndrome—can be explained by the above described inhibition of phosphorylase activity. The opposite disturbances in carbohydrate metabolism occurring in animals after hypophysectomy or adrenalectomy, and in patients with hypopituitarism or Addison's disease, might be explained as a deficiency in the hormonal regulation of the enzyme phosphorylase. Hyperglycemia and increased formation of lactic acid following adrenaline injection might conceivably depend upon stimulation of phosphorylase activity.

After administration of large doses of cortisone or ACTH a number of changes occur in the body which cannot be explained on the basis of phosphorylase inhibition. This suggests that, in the human and animal organism, cortisone or ACTH does not attack phosphorylase alone. Another point of attack, so far not definable in detail, may be the so-called metabolic pool (Albright 1943).

## SUMMARY

Experiments on rabbits have shown that phosphorylase activity in untreated animals is about 50 per cent of the total activity with added muscle adenylic acid. In animals treated with 4–5 mg. cortisone daily for one week the activity was depressed to one-half and with 10–20 mg. to about one-fifth of the original. The enzyme can be completely inactivated with a dose of 10–20 mg. cortisone. The glycogen content of muscle then increases three of four times and that of liver 20–30 times. Cortisone seems to be capable of inhibiting phosphorylase activity, the breakdown of glycogen being more strongly inhibited than its storage.

In experimental animals treated with large doses of cortisone, which according to previous investigations causes phosphorylase activity to fall to very low values, this activity was found to be almost normal after

adrenaline injection. The increase in blood sugar after adrenaline administration was unchanged in such animals, regardless of whether cortisone had produced permanent hyperglycemia or not. Yet, in animals given cortisone, the highest blood sugar value as a rule appeared half an hour later and the blood sugar returned more slowly to the original values. This seems to be due to the fact that the phosphorylase inactivated by cortisone must first be reactivated by adrenaline. The tests seem to show that the organism has at its disposal a hormonally controlled mechanism for the regulation of glycogen storage and breakdown. Cortisone retards phosphorylase activity and renders difficult the synthesis, and especially the breakdown of glycogen. Adrenaline, on the other hand, stimulates phosphorylase, which results in increased glycogenolysis.

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# THE RELATIVE IMPORTANCE OF GENETIC AND ENVIRONMENTAL FACTORS IN DETERMINING THE PRECISION OF GONADOTROPHIN ASSAY

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AT PRESENT, the assay of gonadotrophin must be based on some biological response. According to Bliss (1950), "The inherent precision of the assay may be increased principally by reducing the standard deviations about the dosage response line and less frequently by increasing the slope without change in the standard deviation".

This paper is concerned with determining to what extent individual variation can be decreased by the use of highly inbred animals as compared to random bred (non-inbred) animals. Furthermore, the influence of certain environmental factors on the standard deviation has been studied.

## MATERIALS AND METHODS

Immature female rats stemming from the Long-Evans strain were used; one strain was random bred and the other was the result of 15 or more generations of close inbreeding. The first 3 generations were the result of sire-daughter matings whereas later generations resulted from brother-sister matings. All of the inbred animals stemmed from a single brother-sister mating in the eighth generation of inbreeding.[1] A test for isogeneity of the inbred animals will be described later.

Extracts of equine gonadotrophin assaying between 100 and 1000 I.U. per mg. total solids were used.[2] The dose employed produced an ovarian weight of approximately 30 mg. The procedures for injection and autopsy have been described by Cole and Erway (1941).

The data for both the inbred and random-bred rats were collected during 1949 and 1950. Two or more litters were autopsied on any day that data were gathered. This permitted a statistical test to be made of the importance of variation due to day of autopsy. Between some days there was a highly significant and large component of variance. However, on careful checking of our results it was found that these large components of variance were probably due to differences in dose, due to errors in weighing the hormone,

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[1] Calculating Wright's (1922) Coefficients of Inbreeding and Relationship it was found that the degree of inbreeding was greater than 97 per cent, the relationship within litters was greater than 99 per cent, and the relationship between litter was greater than 94 per cent. The theoretical implication of the high values of these coefficients of inbreeding and relationship is that the genetic variance had been essentially removed from this inbred colony of rats.

[2] We wish to express our appreciation to Dr. Erwin Schwenk of Schering Corporation, Bloomfield, N. J. for supplying extract 35-D testing 1000 I.U. per mg.

since large differences between days did not occur when the same batch of diluted hormone was compared.

In view of the variations obtained with different batches of diluted hormone, the data were analyzed on an intraday basis; that is, the data combined in any analysis were pooled in such a way as to eliminate any effect of day. As only one batch of diluted hormone was used on any one date, the effect of variation in size of dose administered was eliminated. Hence, when we speak of variance we will refer to the variance of a hypothetical population of rats all autopsied on the same date.

In a population stratified according to full sib litters, the variance of individuals ($T$) may be partitioned as follows:

$$T = W + L \tag{1}$$

where $W$ is the variance within litters and $L$ is the variance between true litter means. Sample estimates of $W$ and $L$ are readily obtained by the use of analysis of variance (Snedecor 1946, p. 234). In every case, prior to pooling the sums of squares of several litters to obtain an estimate of $W$, Bartlett's $X^2$ test of homogeneity of the intralitter variances was made (Snedecor 1946, p. 251). In these tests the largest $X^2$ obtained was less than the $X^2$ associated with the 10 per cent level of probability. These homogeneity tests served as a check on experimental technique and also indicated that the intralitter variance was independent of the size of the litter mean.

For a population of rats resulting from random matings

$$W = \tfrac{1}{2}H + E, \qquad L = \tfrac{1}{2}H + C \tag{2}$$

where $H$ is the component of variance due to differences between the hereditary constitutions of individuals, $E$ is the component of variance due to non-genetic (environmentally induced) differences between individuals, $C$ is the component of variance due to environmental conditions that are similar for litter mates but differ from litter to litter.[3]

In a population of rats in which the hereditary variation has been essentially removed, say, through generations of close inbreeding,

$$W = E \quad \text{and} \quad L = C \tag{3}$$

If the variance ($T_i$) of such an inbred population is compared with the variance ($T_r$) of a random breeding population maintained under the same conditions, it follows then that

$$T_r - T_i = H_r, \tag{4}$$

which is the component of hereditary variance in the random breeding population, and $H_r/T_r$ is the fraction of the total variance that is due to hereditary differences between individuals of the random breeding population.

## RESULTS AND DISCUSSION

### *Test for isogeneity of the inbred strain*

The following experimental check on the assumption of the removal

[3] This is the simplest statement of the partitioning of the hereditary variance on an intralitter and interlitter basis (Wright 1921). It is accurate to the extent that non linear gene interactions and non linear hereditary and environmental interactions are unimportant. Chapman (1946) in his study of the weight response of the immature rat ovary to gonadotrophic hormone found non linear gene interactions to be relatively unimportant.

of the genetic variance from the inbred strain was made. Within the inbred strain rats were randomly assigned to dams at birth for rearing. At 21 days these rats were weaned. Those rats raised by a given mother were housed in the same cage together. At 25 days the rats were treated with gonadotrophin and the ovarian response was determined at autopsy 48 hours after injection. An estimate of intralitter variance of ovary weights for these randomized foster litters was made and compared with that for litters of inbred rats left with their own dams. These results are presented in Table 1.

TABLE 1. A COMPARISON OF INTRALITTER VARIANCE OF RATS RANDOMLY ASSIGNED TO DAMS WITH THAT OF RATS LEFT WITH THEIR OWN DAMS

| | Number of rats | Number of litters | Estimate of intralitter variance (W) |
|---|---|---|---|
| Rats randomly assigned to dams | 51 | 10 | 9.11 |
| Rats left with own dams | 179 | 43 | 10.31 |

The extent to which genetic variation remained in the inbred stock should be reflected in a larger intralitter variance for the randomly assigned rats than for those left with their own dams. As can be seen in Table 1, the experimental check is thus compatible with the theoretical expectation that the genetic variance has been essentially removed from the inbred stock.

*Increase in precision of assay due to removal of genetic variance*

The results of the analysis of variation in ovary weight response to gonadotrophin injection for the inbred and randomly bred rats are set forth in Table 2.

TABLE 2. VARIATION IN OVARY WEIGHT RESPONSE TO GONADOTROPHIN INJECTION

| Year | Rats | Number of rats | Number of litters | Average ovary weight in mg. | Estimate of intralitter variance W | Estimate of between litters variance L | Estimate of total variance T = W + L |
|---|---|---|---|---|---|---|---|
| 1949 | Inbred | 80 | 17 | 24.3–35.0* 30.0 | 2.96 | 3.15 | 6.11 |
| | Randomly bred | 49 | 9 | 20.4–35.3 27.0 | 19.91 | 20.73 | 40.64 |
| 1950 | Inbred | 230 | 53 | 28.4–43.7 34.5 | 10.03 | 3.27 | 13.30 |
| | Randomly bred | 96 | 30 | 24.3–48.2 36.8 | 37.74 | 27.35 | 65.09 |

* Maximum and minimum litter averages. The wide range of litter averages reflects the variation in size of dose used on different days as mentioned on page 2.

On perusal of Table 2 one is struck by the larger variance estimates for both the inbred and randomly bred rats in 1950 as compared with 1949. Prior to discussing this point we will consider the results within each year separately.

Substituting the 1949 estimates in equations (1) through (4) we obtain $H_r/T_r = 34.53/40.64 = .85$, or 85 per cent of the variance in the randomly bred rats is estimated to be due to hereditary differences between individuals. It is to be noted that these results are in close agreement with the theoretical expectations (as stated in equations (2) ) that half of the genetic variance is found within litters and the remaining half between the means of litters. Treating the 1950 results similarly we obtain $H_r/T_r = 51.79/65.09 = .80$ or 80 per cent. Once again the results are consistent with the partitioning of the hereditary variance as set forth in equations (2). The consistency of both the 1949 and 1950 results on this latter point implies that hereditary differences between dams are unimportant as a source of maternal influence. If this were not so we would expect a larger $C$ term for the randomly bred rats than for the inbred rats. This would in turn lead to larger values for $L$ relative to $W$ in the randomly bred stock. This implied unimportance of hereditary maternal affects is in agreement with Chapman (1946) who concluded that permanent maternal affects were unimportant.

If $n$ rats are randomly chosen for an assay, the variance of their average response will be $T/n$ (where $T$ is the variance of individual responses) which is a measure of the precision of the response. On the basis of our 1949 results about 7 times as many random bred rats (non-inbred) are needed as inbred rats to achieve a given level of precision. On the basis of the 1950 results the ratio is 5 to 1.

After reviewing the experimental technique employed in the assays, the only plausible hypothesis that presented intself to account for the larger variance estimates in 1950 as compared with 1949 was the following. Experimental conditions in 1949 and 1950 were the same except that in the former years the product injected was 35-D while in the latter year the products were C-17-1 and C-17-2. If it is asssumed that the 35-D went into solution more uniformly than did the C-17-1 and C-17-2, then there would be less variation in actual potency from injection to injection in 1949 than in 1950. However, this assumption was not confirmed by the following experiment performed during December 1951 and January 1952. Unfortunately no 35-D was available. Eighteen inbred litters were divided into four groups according to the material injected. The materials injected were: 1) C-17-1 centrifuged before dilution, 2) C-17-1 not centrifuged before dilution, 3) C-17-2 centrifuged before dilution, 4) C-17-2 not centrifuged before dilution. Intralitter variances were estimated:

| Group | Number of rats | Number of litters | Estimate of Intralitter variance |
|---|---|---|---|
| 1 | 20 | 5 | 2.68 |
| 2 | 20 | 5 | 4.40 |
| 3 | 20 | 4 | 4.07 |
| 4 | 21 | 4 | 3.10 |

There are no significant differences between the intralitter variance estimates for these four groups. Furthermore, the intralitter variance estimate for all of these litters is 3.54 which is not significantly different from the 2.96 estimate for the 1949 inbred rats injected with 35-D but is significantly different from the 1950 inbred results with C-17-1 and C-17-2 (10.03). This experiment suggests that the difference between the 1949 and 1950 variance estimates is probably due to some other cause than the one we tentatively suggested.

*Certain environmental factors influencing precision of assay*

Two factors that might act as causes of environmentally induced variation in ovary weight were considered. These were variation in the consumption of water and feed during the period from injection to autopsy. The following three experiments were conducted during 1950 using inbred rats:

A. *Twenty-four hour fast.* Twelve rats from 3 litters were deprived of feed for 24 hours between the times of injection and autopsy.
B. *Forty-eight hour fast.* Sixteen rats from 3 litters were deprived of feed for 48 hours preceding autopsy.
C. *Forty-eight hour water deprivation.* Sixteen rats from 4 litters were deprived of water for 48 hours preceding autopsy.

Control litters having free access to feed and water were used in each experiment.

The results are set forth in Table 3.

If the amount of feed or water consumed affects ovary weight, then in addition to a difference between mean ovary weights of experimental and control rats we might also expect a decrease in the intralitter variance since deprivation of the factor in question could make for a more uniform environment. This would seem to be born out in the results presented in Table 3, wherein it is seen that in the experiment (48 hour water deprivation) which resulted in the greatest mean difference between the experimental and control rats the greatest decrease in the intralitter variance occurred.

The intralitter variance estimates of the experimental rats in none of the experiments were significantly less than that for the control rats at the 5 per cent level of probability. This lack of significance, especially in the

TABLE 3. SUMMARY OF RESULTS OF FEED AND WATER DEPRIVATION EXPERIMENTS

| Experiment | | Number of rats | Number of litters | Intralitter variance estimate W | Mean ovary weight in mg. | Difference between mean ovary weights of control and experimental rats in mg. |
|---|---|---|---|---|---|---|
| 24 hour fast | Experimental | 12 | 3 | 10.10 | 33.6 | 3.2† |
| | Control | 14 | 3 | 10.03* | 36.8 | |
| 48 hour fast | Experimental | 16 | 3 | 8.49 | 29.4 | 3.1† |
| | Control | 15 | 3 | 10.03* | 32.5 | |
| 48 hour water deprivation | Experimental | 16 | 4 | 4.85 | 27.3 | 10.0‡ |
| | Control | 15 | 4 | 10.03* | 38.3 | |

* The intralitter variance for the controls is based on the overall 1950 trials (see table 2).
† Level of significance $P < .02$.
‡ Level of significance $P < .001$.

case of the water deprivation experiment, is most likely due to the small number of experimental rats involved.

Since the degrees of freedom were so few in estimating the variances of the experimental rats it was decided to use the overall 1950 variance estimate of 13.30 (see Table 2) for both experimental and control rats in computing the standard errors of the differences of the means in Table 3. This variance estimate is based on 219 degrees of freedom. The test of significance of the differences between means is therefore not an exact test. It has the virtue, however, of being conservative in detecting significant differences since a larger variance estimate has been used for the experimental rats than theoretical considerations suggest. Therefore, the significant differences indicated in Table 3 are on the safe side.

Variations in feed and water consumption, below some optimum level perhaps, thus seem to be potential sources of variation in ovary weights, with water being far more important than feed. In the case of water it is obvious that it could contribute to differences between litters if in some cages the supply was accidentally shut off, as, for example, through a clogged water tube. The large decrease in the intralitter variance in the water deprivation experiment if taken at its face value suggests that rats in the same cage may consume water at different rates.

## SUMMARY

A highly inbred line of rats has been developed for gonadotrophin assay based on ovary weights of immature females. Experimental and theoretical evidence indicates that genetic variation in ovary weights has been essentially eliminated in this line. A comparison of variability of the inbred rats with that of random bred rats (non-inbred) indicates that 80 to 85 per cent of the variance of the random bred rats is of genetic origin.

Translated into terms of precision of assay, 5 to 7 times as many random bred rats are needed as inbred rats to achieve a given level of precision.

Variation in feed and water supply during the period between injection and autopsy was found to significantly contribute to the variability of ovary weights. The effect of water was far more important than that of feed.

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# EFFECTS OF ADRENOCORTICOTROPHIC HORMONE ON THE PHOSPHORUS METABOLISM OF LYMPHOID TISSUE*

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THE subcutaneous injection of a single dose of adrenocorticotrophic hormone (ACTH) in mice of the CBA strain produced a series of histological changes in lymphoid tissues characterized by three phases: degenerative, repair, and recovery (Dougherty and White, 1945). The degenerative phase was apparent in one hour and persisted for a six hour period. This phase was characterized by pycnosis of medium sized and small lymphocytes, shedding of cytoplasm of lymphocytes, decreased number of lymphocytes, marked edema, and cessation of mitosis. The repair phase which began at six hours following hormone injection included phagocytosis of nuclear debris and deposition of basophilic material in the cytoplasm of reticular cells. The recovery changes started at approximately nine hours after ACTH administration and were characterized by mitoses in the remaining lymphocytes of lymphoid tissue, maturation of reticular lymphocytes and differentiation of reticular lymphocytes from reticular cells. Within 24 hours after ACTH injection the lymphoid tissue resembled that of normal animals except for the thymus, which was still depleted of lymphocytes.

The profound changes in the histology of lymphoid tissue produced by ACTH might be expected to be accompanied by alterations in the metabolism of these cells. Changes in oxygen consumption were not large,[1] and alteration of adrenal function did not appear to affect the glycolytic activity or amino acid production of normal lymphoid tissue (Roberts and White, 1951; White, 1951).

Gemzell and Samuels (1950) studied the effect of ACTH on the phosphorus metabolism of rats, but did not examine lymphoid tissue. They reported a fall in inorganic phosphorus of the plasma six hours after ACTH

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[1] Roberts, S., and A. White, Unpublished results.

was administered to the normal animal. These same authors found an increased concentration of liver inorganic phosphate and a decreased turnover a muscle inorganic phosphate following adrenalectomy. Geschwind, Li and Evans (1950) demonstrated that treatment of the hypophysectomized animal with ACTH resulted in an increased turnover of liver phospholipids. No significant effect of adrenalectomy or cortisone treatment upon the phosphocreatine or adenosine triphosphate content of rat muscle was observed by Albaum, Hirshfeld, Tonhazy and Umbreit (1951).

The present investigation concerns a study of the changes in absolute concentration of phosphorus and in the incorporation of radioactive phosphorus in the total phosphorus and four phosphorus fractions, namely, acid soluble (A.S.P.), phospholipid (P.L.), ribonucleic acid phosphorus (R.N.A.P.) and desoxyribonucleic acid phosphorus (D.N.A.P.), of lymphoid tissue in CBA mice following adrenalectomy, and as a consequence of ACTH injection.

## METHODS

Male mice of the CBA strain (Strong) eight weeks old and weighing 19–25 grams were used. In the adrenalectomized group, operation was performed seven days prior to study of the animals, during which period they received 0.9% saline as drinking fluid. Completeness of the operation was determined by noting at autopsy the absence of adrenal tissue and the hypertrophy of thymus and lymph nodes.

In the studies with intact mice, ACTH (Armour) was dissolved in 0.2 ml. of physiological saline and injected subcutaneously in a dose of one mg. per mouse. Eight microcuries $P^{32}$ in 0.2 ml. of 0.001 M phosphate buffer (pH 7.0) was injected subcutaneously in two groups of experiments. In one, the $P^{32}$ was given 72 hours before ACTH injection, while in the other it was given three and one-half hours after ACTH injection and two and one-half hours prior to autopsy. In the former study, the net reaction was largely loss of $P^{32}$ from the tissue, and in the latter, the net reaction was largely incorporation of phosphorus, although within two and one-half hours after $P^{32}$ administration certain fractions (A.S.P.) were past their maximum specific activity, and others (R.N.A.P.) were near their maximum (Barnum and Huseby, 1950).

At appropriate periods of time, groups of animals were sacrificed by severing the cervical vertebrae. Lymph nodes (axillary, inguinal, and mesenteric), thymus and spleen were removed, blotted and placed in a chilled petri dish until autopsy of all animals in the group was completed. The pooled samples were then weighed and frozen with the aid of dry ice. The samples were kept frozen until homogenized with trichloroacetic acid and fractionated according to Schneider's modification (Schneider, 1946) of the Schmidt and Thannhauser procedure. Aliquots of the fractions were used for total phosphorus determination by the method of Fiske and Subbarow as described by Umbreit, Burris and Stauffer (1951). $P^{32}$ determinations were made on other aliquots with an end window tube and Tracerlab scaler.

## RESULTS

Administration of ACTH to the normal animal, beginning at 72 hours following the injection of radioactive phosphorus, did not alter significantly

the concentration of total phosphorus or the incorporation of $P^{32}$ in animals autopsied at 1, 6 and 24 hours after hormone injection (Table 1). The results of phosphorus fractionation show only small changes in concentration or in uptake of phosphorus in all of the fractions studied (Table 2).

TABLE 1. EFFECT OF ACTH ON CONCENTRATION OF TOTAL PHOSPHORUS* AND ON TURNOVER† OF TOTAL PHOSPHORUS, EXPRESSED AS MEAN PER CENT DEVIATION FROM CONTROLS‡

| Tissue | Time after ACTH | | | | | |
|---|---|---|---|---|---|---|
| | 1 hr. | | 6 hrs. | | 24 hrs. | |
| | Total P | $P^{32}$ | Total P | $P^{32}$ | Total P | $P^{32}$ |
| Thymus | +9.6 | − 5.0 | −7.6 | −7.8 | +2.6 | −3.3 |
| Nodes | +1.1 | +11.0 | +1.9 | −7.9 | +3.6 | +3.8 |
| Spleen | −5.4 | − 7.4 | +8.3 | −2.2 | +8.7 | −5.2 |

* Per cent difference $= 100 \dfrac{(\text{Mg P/100 g. Exp.} - \text{Mg/100 g. control})}{\text{Mg. P. 100 g. control}}$

† Per cent difference $= 100 \dfrac{(\text{Per cent dose/g. Exp.} - \text{Per cent dose/g. control})}{\text{Per cent dose/g. control}}$

‡ For each time period there were six control and six hormone treated animals, with individual analysis of each tissue in each animal.

TABLE 2. EFFECT OF ACTH ON PHOSPHORUS FRACTIONS OF THYMUS, LYMPH NODES, AND SPLEEN OF MICE. VALUES GIVEN ARE MEAN PER CENT DEVIATIONS OF CONCENTRATION* AND TURNOVER† OF PHOSPHATE FRACTIONS

| Fraction | Thymus | | | Nodes | | | Spleen | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1hr.§ | 6 hrs.‖ | 24 hrs.§ | 1 hr.§ | 6 hrs.‖ | 24 hrs.§ | 1 hr.§ | 6 hrs.‖ | 24 hrs.§ |
| ASP | − 3.2 | − 0.8 | − 3.0 | + 3.8 | − 5.6 | +0.3 | +10.6 | + 7.0 | +11.6 |
| $ASP^{1}$‡ | + 1.5 | − 2.4 | − 5.0 | − 8.5 | + 2.1 | +6.1 | − 7.7 | − 6.7 | + 9.8 |
| PL | − 7.0 | + 8.0 | + 3.4 | −11.0 | − 7.7 | −5.7 | + 7.5 | + 0.4 | − 8.0 |
| $P^{1}L$ | −10.6 | +11.0 | + 5.0 | − 8.8 | + 4.8 | +5.0 | −11.3 | + 0.9 | + 4.0 |
| RNAP | + 2.4 | − 6.7 | − 9.4 | + 5.6 | + 3.0 | −7.5 | −10.2 | −11.4 | − 5.7 |
| $RNAP^{1}$ | − 0.6 | − 5.4 | − 7.3 | + 2.1 | + 6.5 | +1.3 | − 6.8 | −10.6 | − 3.3 |
| DNAP | + 1.2 | + 5.0 | −10.1 | − 1.0 | − 5.6 | −2.6 | − 5.5 | −11.0 | + 5.3 |
| $DNAP^{1}$ | − 3.5 | + 5.3 | −12.2 | −11.2 | − 6.9 | −5.9 | −12.4 | − 8.3 | − 0.9 |

* Same as Table 1.
† Same as Table 1.
‡ $P^{1}$ indicates labeled phosphorus data.
§ Two control groups of 10 animals each; two treated groups of 10 animals each.
‖ Four control groups of 10 animals each; four treated groups of 10 animals each.

In experiments in which ACTH was given first, six hours prior to autopsy, and the $P^{32}$ two and one-half hours before autopsy, definite changes were observed in the extent of incorporation of $P^{32}$ (Table 3). The phospholipid fraction of the thymus showed the largest changes. The increased incorporation of $P^{32}$ in the spleen R.N.A. tends to support the suggestion

of a different effect of ACTH on the thymus and lymph nodes as compared with that found in spleen. This is also substantiated by measurements of the specific activity of the $P^{32}$ in purified (dissolved in alkali and reprecipitated with trichloroacetic acid three times) D.N.A. The per cent deviation from controls of the specific activity for the D.N.A. was thymus, −19.9; nodes, −23.0; and spleen, −2.0. The values obtained in the other phosphorus fractions of the experimental animals did not differ significantly from the controls.

Following adrenalectomy, no differences were found in the concentration or turnover of $P^{32}$ in lymphoid tissue when the $P^{32}$ was given 72 hours before autopsy. This is in agreement with the findings of Sternberg and LeBars (1949), who autopsied animals 48 hours after $P^{32}$ injection.

The variations experienced in these experiments leave much to be desired. Schultz (1950) has noted the need for more adequate methods for nucleic acid analysis. Barnum and Huseby (1950) have cited the desirability of fasting animals 16 hours or more before injection of $P^{32}$. Since fasting for 16 hours initiates considerable stress in mice, it was deemed advisable to use fed, treated mice and to compare these with suitable controls. In

TABLE 3. EFFECT OF ACTH ON INCORPORATION* OF $P^{32}$ INTO PHOSPHATE FRACTIONS. VALUES GIVEN ARE MEAN PER CENT DEVIATIONS FROM CONTROLS. ACTH GIVEN SIX HOURS, AND $P^{32}$ TWO AND ONE-HALF HOURS, BEFORE AUTOPSY. 40 CONTROL AND 40 TREATED ANIMALS IN FOUR SEPARATE EXPERIMENTS, 10 ANIMALS IN EACH GROUP

| Fraction | Thymus | Nodes | Spleen |
|---|---|---|---|
| $ASP^{i}$† | −15.7 | − 2.5 | − 7.8 |
| $P^{i}L$ | −38.5‡ | −20.6 | − 9.6 |
| $RNAP^{i}$ | −27.9‡ | −33.8‡ | +33.4‡ |

* Same as Table 1, footnote.†

† $P^{i}$ = radioactive phosphorus data.

‡ Significant values, i.e., greater than 2.5 times the square root of the sum of the squares of the standard errors of control and experimental groups.

this way it was hoped to minimize all effects other than those due to ACTH. Mice of the CBA strain were employed since histological and other metabolic effects in these animals have been studied in some detail (Dougherty and White, 1945, 1947; White, 1949). It is recognized that untreated mice are very susceptible to alterations in external environment leading to "stress" responses and hence may not always be strictly controls. The mean values for the concentration of phosphorus in all the fractions in control animals in this study compare favorably with those listed for rats by Schneider and Klug (1946) and also for nucleic acids with those given by Davidson (1947).

## DISCUSSION

The lack of an effect of ACTH on the concentration of phosphorus or on the net incorporation of $P^{32}$ when the $P^{32}$ was given 78 hours before autopsy, coupled with an effect when the $P^{32}$ is given two and one-half hours before autopsy, viewed together with the histological picture (Dougherty and White, 1945), may be given several interpretations. There may have occurred significant changes in the concentration of certain phosphorus fractions and the inhibition of $P^{32}$ incorporation could be due at least in part to a decreased concentration of phosphorus. However, the decreased specific activity of the purified D.N.A. suggests that, at least in this phosphorus fraction, this is not an acceptable explanation. It may also be postulated that the inhibition of $P^{32}$ turnover following hormone injection is due largely to diminished incorporation of $P^{32}$ into the cell. This inhibition is not as apparent when the $P^{32}$ was given 78 hours before autopsy since the specific acitivity of the inorganic phosphorus was low. Furthermore the design of the experiment with the $P^{32}$ given 72 hours before ACTH was such as to emphasize the degradative phase. The data in this group of experiments suggest that the mechanism for the increased dissolution of lymphoid tissue, following ACTH administration, is not one of a generalized acceleration of phosphorus turnover.

The effects seen when ACTH was given six hours, and $P^{32}$ two and one-half hours, prior to autopsy are consistent with the suggestion that one of the actions of ACTH on lymphoid tissue metabolism is an inhibition of incorporation of phosphorus into lymphoid cells. This is in accord with a decrease in $P^{32}$ incorporation with little change in concentration of phosphorus, and with the apparent capacity of ACTH to cause an early inhibition of mitosis in lymphocytes, as seen histologically.

The *in vitro* experiments of Hechter and Johnson (1949) suggested that eosin resistant cells become eosin stainable prior to dissolution, and that the percentage of non-viable eosin stainable cells in a suspension at a particular time is the resultant of the rates of conversion of dye resistant to dye stainable cells, and the dissolution of the latter. Similarly, *in vivo* the number of cells in which the $P^{32}$ incorporation is inhibited is the resultant of the conversion of normal cells to cells in which inhibition of $P^{32}$ uptake occurs, and the dissolution of these cells. This leaves the remaining tissue normal in turnover and mitotic activity (Stoerk and Solotorousky, 1950).

The histological finding of an inhibition of mitosis, concomitant with an inhibition of $P^{32}$ incorporation into D.N.A., is supporting evidence for the postulate of Hammersten and Hevesy (1946) that phosphate ion enters D.N.A. only at the time of mitosis. It is of interest that cortisone has

been reported to inhibit the incorporation of labeled formate by both D.N.A. and R.N.A. in the viscera of mice (Skipper *et al.*, 1951). However, since inhibition of $P^{32}$ uptake in ribonucleic acid and phospholipid also occurred in the present study, it is apparent that more than an inhibition of mitosis must be assigned to the effects of ACTH. Phosphate incorporation into fractions other than D.N.A. occurs in preparations not undergoing cell division (Friedkin and Lehninger, 1949). Umbreit and Tonhazy (1951) have emphasized that cortisone produces a variety of metabolic effects, rather than a single more or less specific alteration in metabolism. The present data are consistent with that concept, and do not preclude the existence of alternate hypotheses to explain the dissolution of lymphoid cells in thymus and nodes as a consequence of ACTH administration. The inhibition of mitosis may be the resultant of other effects, rather than an independent reaction.

Definite proof that the inhibition *in vivo* is in those cells undergoing dissolution is not given by the retarded $P^{32}$ turnover seen in these experiments. The histological effects of ACTH are seen most strikingly in medium sized and small lymphocytes (Dougherty and White, 1945). The concept of an inhibition of $P^{32}$ incorporation prior to dissolution of cells is not necessarily new. The blocking of specific reactions in the synthesis of phospholipids, ribonucleic acids and desoxyribonucleic acids in all cells for a certain interval of time, with subsequent recovery of activity, is an alternate and acceptable mechanism for the observed inhibition.

Clearly a different mechanism of action on the nucleic acid phosphorus metabolism of the rat seminal vesicle must be ascribed to testosterone (Rabinovitch, Junqueira and Rothschild, 1951), since this hormone increased significantly the absolute concentration of ribonucleic acid phosphorus. This finding is in harmony with the contrasting effects of ACTH and testosterone on nitrogen metabolism.

## SUMMARY

(1) When ACTH was given to mice of the CBA strain, no changes were found in the concentrations of total phosphorus or in four fractions of the total phosphorus of thymus, lymph nodes or spleen.

(2) No alterations in phosphorus turnover, as measured by $P^{32}$, were observed when the $P^{32}$ was given 72 hours prior to the hormone.

(3) Inhibition of incorporation of $P^{32}$ into phospholipid, ribonucleic acid and desoxyribonucleic phosphorus was found when ACTH was injected six hours, and $P^{32}$ two and one-half hours, prior to autopsy.

(4) The relationships between histological and chemical findings are considered to support the general conclusion that the inhibition of $P^{32}$ turnover, seen acutely following ACTH injection, is a reflection in part of

the inhibition of mitosis of lymphocytes caused by hormone administration.

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# INFLUENCE ON THE REPLACEMENT VALUE OF SOME ADRENAL-CORTEX STEROIDS OF DIETARY SODIUM AND SYNERGISM OF STEROIDS IN LACTATING ADRENALECTOMIZED RATS

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GAUNT, Eversole and Kendall (1942) reported that 11-dehydrocorticosterone and 17-hydroxy-11-dehydrocorticosterone (cortisone) were more effective than desoxycorticosterone acetate (doca) in maintaining lactation in adrenalectomized rats and it was concluded that the critical factor for the maintenance of normal lactation in the adrenalectomized rat was a sufficiency of the 11-oxygenated cortical steroids which were then believed to be mainly concerned with protein and carbohydrate metabolism. Folley and Cowie (1944), however, were unable to confirm these observations. They found that doca, while not completely restoring lactation to the normal level, was superior to either of the above 11-oxygenated steroids. Later, Cowie and Folley (1947 a) confirmed and extended their earlier results and then attempted to enhance the replacement value of the 11-oxygenated steroids by increasing the protein content of the diet from 20% to 50% (Cowie and Folley, 1947 b). These attempts were unsuccessful.

Kendall (1946) suggested that the dietary intake of sodium and potassium might have a secondary effect on the action of the 11-oxygenated steroids on gluconeogenesis and that differences in the sodium and potassium contents of the rat diets might be the reason for the discordant results obtained by the two groups of workers. The first two experiments described below were undertaken to investigate such a possibility. Unfortunately, owing to the impossibility of obtaining a sufficient supply of cortisone when these experiments were carried out, only 11-dehydrocorticosterone and its acetate were available for trial. For comparative purposes groups of rats were also treated with doca. It will be seen that these experiments failed to clarify the situation and when cortisone again became available two further experiments were carried out to find whether the

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efficacy of cortisone could be enhanced by administering it by the tablet-implantation technic and whether doca, when administered in conjunction with cortisone, would have a synergistic action in the restoration of lactation.

The first experiment relating to changes in dietary sodium has already been reported in abstract (Cowie, Folley, French and Greenbaum, 1947).

## EXPERIMENTAL

### *Materials and methods*

The rats (primiparous females, 4 to 5 months old) and the general procedures used have been described in previous papers (Cowie and Folley, 1947 a, b). The particulars of the diets are as follows:

*Experiment 1.* The control diet used was the old stock-colony diet to which dried whole milk had been added in the proportion of 375:1,000 as in a previous experiment (Cowie and Folley, 1947 b). This control diet contained 0.33% sodium and 0.88% potassium. The high-sodium diet (No. 1) was this control diet to which sodium chloride (analytical reagent standard) had been added to give a sodium content of 0.67%.

*Experiment 2.* The control diet was similar to the control diet in experiment 1, the sodium and potassium contents being 0.34% and 0.82% respectively. The high-sodium diet (No. 2) was this control diet with sodium chloride added to give a sodium content of 1.04%.

*Experiments 3 and 4.* The diet used in these experiments was the new stock-colony diet (see Cowie and Folley, 1948) to which dried whole milk had been added in the proportion of 375:1,000.

In all the above experiments the supplements of raw liver and vegetables were fed as in previous experiments (Cowie and Folley, 1947 a, b).

Adrenalectomy was carried out on the fourth day of lactation, the rats in the control groups being subjected to a sham operation. The operative technic used has already been described (Cowie, 1949).

In experiment 1, the adrenal steroids were administered in sesame oil by subcutaneous injection so that the daily dose of the steroid was contained in one injection of 0.5 cc. oil. In experiments 2 and 3, the sesame oil was replaced by arachis oil, as the former had become unobtainable, and two injections per day were given of 0.25 cc. oil. In experiments 1 and 2, the control groups of rats received injections of the appropriate oil by itself. Full particulars regarding the quantities of steroids given will be found in Tables 1 and 2.

The tablets of cortisone and doca used in experiments 3 and 4 were prepared in the laboratory from the crystalline steroids by the method described by Cowie and Folley in 1946. They were made by compression and contained no diluent or excipient. The cortisone tablets weighed approximately 15 mg., were 3.0 mm. in diameter, and were prepared with a total pressure on the tablet of 0.18 ton (183 kg.). At the termination of experiment 3 these tablets were recovered, cleaned, dried and weighed. They were then stored and used again in experiment 4, their weight now being about 11 mg. The tablets of doca for experiment 4 weighed approximately 50 mg. and were subjected in preparation to a total pressure on the tablet of 0.37 ton (376 kg.). All the tablets were cylindrical in form.

The paired feeding of groups in experiments 2 and 4 was carried out as described by

Cowie and Folley (1948). In experiments 3 and 4 the daily food consumption of all rats was measured.

In experiments 1 and 2, the mothers and litters were killed on the 17th day of lactation, in experiments 3 and 4 on the 14th to 16th days. These times were chosen to allow various biochemical studies to be made on the mammary glands and other organs. Some of the biochemical investigations have already been reported (Folley and Watson, 1951).

TABLE 1. DETAILS OF TREATMENTS IN EXPERIMENTS 1 AND 2 AND RESPONSES TO REPLACEMENT THERAPY

| Treatments | No. of mothers | Mean litter-growth indices‖ gm./day with S.E. | Mean percentage change in weight of mothers between 4–14th days with S.E. |
|---|---|---|---|
| Experiment 1 (August, 1946) | | | |
| *Sham-operated rats* | | | |
| No treatment, on stock diet* | 6 | 16.7±0.9 | +12.5±2.3 |
| No treatment, on high-sodium diet (No. 1)† | 6 | 16.9±0.5 | + 9.9±1.9 |
| *Adrenalectomized rats* | | | |
| No treatment, on stock diet | 6 | 8.2±1.1 | + 3.0±1.9 |
| No treatment, on high-sodium diet (No. 1) | 6 | 8.4±0.7 | + 8.3±2.3 |
| 0.56 mg. doca/day, on stock diet | 6 | 10.7±1.0 | +18.5±1.5 |
| 0.56 mg. doca/day, on high-sodium diet (No. 1) | 6 | 11.6±0.5 | +21.1±1.9 |
| 0.56 mg. 11-dehydrocorticosterone acetate/day, on stock diet | 6 | 9.4±0.9 | + 4.6±1.9 |
| 0.56 mg. 11-dehydrocorticosterone acetate/day, on high-sodium diet (No. 1) | 6 | 9.5±1.1 | + 5.2±2.8 |
| 3.0 mg. doca/day, on stock diet | 6 | 13.0±0.8 | +22.9±1.3 |
| 3.0 mg. doca/day, on high-sodium diet (No. 1) | 6 | 13.4±0.9 | +21.2±1.8 |
| Experiment 2 (May–June, 1947) | | | |
| *Sham-operated rats* | | | |
| No treatment, on stock diet‡ | 8 | 15.3±0.7 | + 8.2±1.1 |
| Pair-fed controls, on stock diet | 15 | 8.8±0.6 | −18.0±2.0 |
| *Adrenalectomized rats* | | | |
| No treatment, on stock diet | 15 | 6.4±0.6 | − 1.2±0.8 |
| 0.56 mg. doca/day, on stock diet | 7 | 10.0±0.5 | +14.7±2.9 |
| 0.56 mg. doca/day, on high-sodium diet (No. 2)§ | 7 | 9.7±0.5 | +15.0±1.6 |
| 0.5 mg. 11-dehydrocorticosterone/day, on stock diet | 7 | 8.7±0.8 | + 2.4±1.5 |
| 0.5 mg. 11-dehydrocorticosterone/day, on high-sodium diet (No. 2) | 7 | 10.4±0.4 | +10.1±2.1 |
| 3.0 mg. doca/day, on stock diet | 10 | 13.1±0.5 | +15.5±1.1 |
| 0.56 mg. doca/day +0.5 mg. 11-dehydrocorticosterone/day, on stock diet | 7 | 11.0±0.6 | +16.0±2.1 |

* This diet contained 0.33% sodium, 0.88% potassium.
† This diet contained 0.67% sodium, 0.88% potassium.
‡ This diet contained 0.34% sodium, 0.82% potassium.
§ This diet contained 1.04% sodium, 0.80% potassium.
‖ The litter-growth index of a group of rats is defined as the mean daily gain in weight per litter over the 5-day period from the 6th to the 11th days (Cowie and Folley, 1947c).

## RESULTS

The litter-growth indices (see Cowie and Folley, 1947 c) and particulars of body-weight changes in the mothers for experiment 1 are set out in Table 1. The partial but not complete restoration of lactation with doca was observed as in previous experiments. 11-dehydrocorticosterone ace-

tate in the dosage used (0.56 mg. per day, equivalent to 0.5 mg. free steroid) had no significant beneficial effect. The lactational responses of the groups of rats on the high-sodium diet (No. 1) were in no instance significantly better than the responses of the corresponding group on the stock diet. In view of these negative results on the responses to steroid administration of the doubling of the sodium content of the diet, a further experiment (experiment 2) was set up in which the sodium content of the experimental diet was increased to three times that of the control diet. The results of experiment 2 are set out in Table 1. In this experiment the effect of adrenalectomy on lactation was somewhat more severe, resulting in a litter-growth index of 6.4 gm. per day as against 8.2 gm. per day in the previous experiment. 11-dehydrocorticosterone, moreover, had a decided beneficial action, the litter-growth index for the group being $8.7 \pm 0.8$ gm. per day which differs significantly from the value for the untreated adrenalectomized group ($P = 0.03$). There may have been a slight enhancement of this effect on the high-sodium diet (No. 2) but the difference is not significant ($P = 0.07$). Indications of a graded response to the two dose-levels of doca were again present both in this experiment and in the first experiment,—this graded relation between dose and response has been discussed fully in a previous paper (Cowie and Folley, 1947 a). No synergism was noted when the low dose of doca (0.56 mg. per day) was given in conjunction with the 11-dehydrocorticosterone, the litter-growth index for the group on the combined treatment being similar to that of the group on the low dose of doca ($11.0 \pm 0.6$ gm. per day as against $10.0 \pm 0.5$ gm. per day).

Marked increases in the body weight of the doca-treated rats, as noted in previous experiments (Cowie and Folley, 1947 a), was evident in both the above experiments. It is likely that much of this increment is due to water retention (Folley and Greenbaum, 1948). The increases in the sodium content of the diets had no significant effects on the magnitude of these increments. In experiment 1, 11-dehydrocorticosterone had no apparent influence on the body weights of the mothers but in experiment 2, the group of animals on the high-sodium diet (No. 2) receiving this steroid showed an increase in weight comparable to that of the control group.

In experiment 3, lactating rats were adrenalectomized on the 4th day and implanted subcutaneously with either one or three 15 mg. tablets of cortisone. The mean daily absorption of cortisone as calculated from the loss in weight of the tablet(s) was $0.36 \pm 0.01$ mg. per day in the animals implanted with one tablet and $1.22 \pm 0.04$ mg. per day in the case of those with three tablets. It will be seen from Table 2 that this treatment was beneficial, the litter-growth indices of the groups receiving one and three tablets of cortisone being similar to the indices of the groups receiving

injections of 1.0 and 3.0 mg. doca daily in oily solution. It is interesting to note that cortisone had no effect on the body weights of the mothers whereas doca gave the usual large increments. Appetite, however, may have been restored to a greater degree with doca (see Table 2).

In view of this beneficial action of cortisone, experiment 4 was carried out to study the efficiency of combined doca and cortisone implants in

TABLE 2. DETAILS OF TREATMENTS IN EXPERIMENTS 3 AND 4 AND RESPONSES TO REPLACEMENT THERAPY

| Treatments | No. of mothers | Mean litter-growth indices* gm./day with S.E. | Mean total food consumption (gm.) between 4-14th days with S.E. | Mean percentage change in weight of mothers between 4–14th days with S.E. |
|---|---|---|---|---|
| Experiment 3 (November, 1950) | | | | |
| | | *Sham-operated rats* | | |
| No treatment | 9 | 13.9±0.7 | 328.4±11.3 | +5.3±0.9 |
| | | *Adrenalectomized rats* | | |
| No treatment | 11 | 4.1±0.6 | 182.7± 8.8 | −3.1±1.9 |
| Cortisone (1×15 mg. tablet =0.36±0.01 mg./day) | 6 | 9.0±0.5 | 240.6±18.1 | −3.5±1.7 |
| Cortisone (3×15 mg. tablets =1.22±0.04 mg./day) | 7 | 10.6±1.1 | 255.1±16.4 | −3.9±1.9 |
| Doca (1 mg./day) | 6 | 9.2±0.8 | 268.9±20.2 | +18.3±2.7 |
| Doca (3 mg./day) | 7 | 10.5±0.4 | 276.9± 9.5 | +12.8±2.6 |
| Experiment 4 (July–August, 1951) | | | | |
| | | *Sham-operated rats* | | |
| No treatment | 8 | 15.6±0.5 | 333.0±11.1 | +8.9±0.8 |
| Pair-fed controls | 8 | 10.1±0.8 | 215.7±11.7 | −14.5±2.2 |
| | | *Adrenalectomized rats* | | |
| No treatment | 9 | 7.5±0.6 | 215.7±11.7 | −2.6±1.1 |
| Doca (1×50 mg. tablet =0.36±0.01 mg./day) | 8 | 12.2±0.5 | 287.1±10.6 | +14.7±2.0 |
| Cortisone (2×11 mg. tablets =0.68±0.05 mg./day) | 7 | 9.9±0.2 | 249.9± 9.7 | −3.2±1.3 |
| Cortisone (2×11 mg. tablets =0.85±0.04 mg./day)+ doca (1×50 mg. tablet =0.36±0.01 mg./day) | 7 | 14.9±0.6 | 310.4±15.9 | +9.3±1.7 |

* The litter-growth index of a group of rats is defined as the mean daily gain in weight per litter over the 5-day period from the 6th to the 11th days (Cowie and Folley, 1947c).

maintaining lactation in adrenalectomized rats. The results are set out in Table 2. In this experiment the response to cortisone (mean daily absorption = 0.68 ± 0.05 mg. per day) was less than in experiment 3. The response to the implants of doca (daily absorption = 0.36 ± 0.01 mg. per day) was good, but with the combined implants of doca and cortisone lactation was apparently maintained at practically the normal level, the litter-growth index of the group being 14.9 ± 0.6. gm. per day as against 15.6 ± 0.5 gm. per day for the control group. The mean daily absorption of steroids from the implants was 0.36 ± 0.01 mg. doca and 0.85 ± 0.04 mg.

cortisone. The figures relating to the per cent change in body weight of the mothers are set out in Table 2. As in experiment 3, cortisone by itself had no apparent effect, but when given in combination with doca it inhibited the occurrence of the large increment in body weight which is regularly associated with the administration of this steroid. The appetite of the mothers on the combined doca-cortisone therapy was restored to normal during the period of treatment (see Table 2).

## DISCUSSION

The sodium and potassium contents of the diet used by Gaunt, Eversole and Kendall (1942) in their experiments on the maintenance of lactation in adrenalectomized rats were 0.54% and 0.58% respectively (Gaunt, 1945). Thus the sodium content of the diets used in experiments 1 and 2 ranged above and below this value for sodium. The results of experiments 1 and 2 seem then to indicate that differences in the dietary sodium are probably not playing a substantial causative role in the varying results obtained by Gaunt, Eversole and Kendall on the one hand and Folley and Cowie on the other. The level of potassium in the diet in the present experiments and in the earlier experiments of this series (Folley and Cowie, 1944; Cowie and Folley, 1947 a, b) is higher than in the diet used by Gaunt *et al.* (1942). It has not been ascertained, however, if this difference affected the issue as it was not possible to lower the potassium contents of the diets in experiments 1 and 2 without completely altering their composition. Nagareda and Gaunt (1948) have briefly reported on the effects of extreme variations in the dietary sodium and potassium on lactating rats adrenalectomized on the first day of lactation. They found that the level of lactation was low on a high-potassium sodium-free diet or high-salt low-potassium diet, but whereas doca improved lactation in adrenalectomized rats on a high-potassium sodium-free diet it had little or no effect on those receiving a high-salt low-potassium diet. While it is thus possible that on account of the higher content of potassium in the diets used in this colony, doca might be expected to be more efficacious, it is doubtful whether the results of such extreme experimental variations in sodium and potassium are applicable to the experiments under discussion where the variations in potassium content were relatively small. In sum, it seems unlikely that differences in the electrolyte content of the rat diets could account for the above-mentioned experimental differences, at least in so far as treatments with doca and 11-dehydrocorticosterone are concerned.

While cortisone had undoubtedly a beneficial effect on the lactational level in experiments 3 and 4, in no case was complete restoration of lactation obtained, neither were the levels of food consumption nor body

weights returned to normal. This observation of the effect of cortisone on lactation and body weight is in accord with the earlier observations of Folley and Cowie (1944) and Cowie and Folley (1947 b). Since food-consumption data were not collected in the earlier experiments a comparison on that aspect cannot be made. In striking contrast to the lack of any synergistic action between doca and 11-dehydrocorticosterone (see experiment 2) is the apparent complete restoration of lactation, appetite and body-weight changes to their normal levels obtained by the combined use of doca and cortisone. In view of this finding further experiments are desirable to determine the effect of increasing the sodium content of the diet of cortisone-treated adrenalectomized rats.

Since doca can effect considerable restoration of lactation in the adrenalectomized rat, indeed complete replacement in certain instances (Cowie and Folley, 1947 a; Smith, 1949) it is surprising that the administration of sodium chloride either in the food or drinking water had by itself little beneficial effect on the restoration of lactation (see experiment 1, and previous experiments described by Cowie and Folley, 1948) and it therefore seems likely, as has been suggested by Folley (1950), that doca must act on the metabolic processes of milk secretion in other ways than by regulating the electrolytic balance. It would be unwise, however, to regard the complete restoration of lactation by doca alone or by the combined use of doca and cortisone as a physiological replacement. There is a growing belief that doca is not a natural product of the adrenal and may not circulate in significant quantities in the body, its actions being pharmacological rather than physiological (see review by Sayers, 1950). Moreover, in addition to this question as to whether doca is normally found in the circulation, biochemical studies carried out on the mammary glands of the rats maintained on the combined therapy indicated that at least some of the metabolic activities of these glands were abnormal. These biochemical studies will be published separately but are mentioned here as they are pertinent to the question as to whether the restoration of lactation in experiment 4 was completely normal.

The results of the paired feeding of groups in experiments 2 and 4 are in agreement with the previous results of Cowie and Folley (1948) and Cowie, Folley, French and Greenbaum (1948), these groups being included for subsequent biochemical study.

A phenomenon of some interest with regard to the absorption of material from subcutaneous implants was observed in experiment 4. When two tablets of cortisone were implanted alone, the total daily absorption from the two tablets was $0.68 \pm 0.05$ mg. per day, but if a tablet of doca was implanted at the same time the absorption from the cortisone tablets was $0.85 \pm 0.04$ mg. per day which is significantly higher. The rate of

absorption of the doca tablets was unaffected by the presence of the cortisone tablets. It seems probable that this increase in the rate of absorption was brought about by alterations in the hydration of the subcutaneous tissues or changes in the local circulation due to the presence of the doca implants.


## SUMMARY

A twofold increase in the sodium content (from 0.33% to 0.67%) of the diet did not affect the replacement value of doca (0.56 or 3.0 mg. per day) or of 11-dehydrocorticosterone acetate (0.56 mg. per day) in maintaining lactation in adrenalectomized rats. A threefold increase in the dietary sodium (to 1.04%) was also without effect on the replacement value of doca (0.56 mg. per day) or 11-dehydrocorticosterone (0.5 mg. per day). In one experiment 11-dehydrocorticosterone acetate gave no lactational response in rats adrenalectomized on the fourth day of lactation; in the second experiment a slight but significant beneficial response was noted with the equivalent dose of 11-dehydrocorticosterone (0.5 mg. per day). No synergistic effect was observed when 11-dehydrocorticosterone (0.5 mg. per day) was given in conjunction with doca (0.56 mg. per day).

Partial restoration of lactation was obtained in adrenalectomized rats by implanting cortisone (1 or 3×15 mg. tablets). Complete restoration of lactation, as estimated from the growth of the litters, was obtained when cortisone (2×11 mg. tablets) was implanted with doca (1×50 mg. tablet). Appetite and body weight were also restored virtually to normal.

Cortisone implants were absorbed somewhat more rapidly when the animal was also implanted with doca.



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# ANDROGEN CONTENT OF TESTIS AND ADRENAL OF WHITE LEGHORN COCKERELS[1]

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CONSIDERABLE attention has continued to be paid in the last few years to the problem of embryonic secretion of sex hormones, among others by Burns, Jost, Moore, and Raynaud in mammals and by Wolff in birds. A second problem, not necessarily directly related to the first, the time of the first secretion of sex hormones in postembryonic life, has also received the interest of several investigators. In poultry, the studies of Breneman (1941, 1950), Ludwig and Boas (1950), and Breneman and Mason (1951) indicate that androgen secretion in White Leghorn cockerels begins about the 10th day after hatching, if not earlier. This conclusion is based upon comb growth observations and is supported by comb growth curves for the Light Brown Leghorn published by Taber (1949, 1951), who states (1949, p. 251) that "It is generally agreed that the chick testis begins secreting androgen during the first few weeks of age. . . . " In the Rhode Island Red cockerel, comb growth is considerably slower than in the White Leghorn (Breneman, 1941); hence, it is possible that the onset of testicular function may vary in different strains of fowl. Kumaran and Taylor (1949) described birefringent materials, presumably testis hormone or its precursors, in the testis of the White Plymouth Rock as early as the 12th day of embryonic life. Severinghaus (1942) well emphasized that hormone production and hormone secretion are to be distinguished; thus, the testis may contain testoid without releasing it.

The present investigation was undertaken primarily to determine if male hormone is *produced* in the testis and/or adrenal of immature single-comb White Leghorn cockerels. Three methods have been employed in an attempt to attain this end:

(1) The use of the 3-day White Leghorn chick comb method for bioassay;
(2) The use of the castrate rat seminal vesicle as a similar target organ for bioassay;
(3) The use of the dinitrophenylhydrazine reaction to determine localization of hormone or associated substances.

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Secondarily, we have been interested in the specific utility of the chick comb as a target organ in determining local response to androgen-containing transplants.

## MATERIALS AND METHODS

### 1. *The Chick Comb as a Bio-indicator of Androgenic Activity*

Ruzicka (1935) first suggested using the chick comb as a bio-indicator of androgenic activity. Frank and Klempner (1937) used 6-day chicks as test animals for qualitative androgen determination. Since that time many modifications have been suggested. Frank *et al.* (1942) published detailed information for androgen assay by the chick comb method which is quite satisfactory and which has been adapted for this study.

A total of 2331 White Leghorn cockerels was used in 1950–1 as series A of this study: 468 cockerels served as donors and 1863 as hosts. The stock was pure Walls strain. The donor cockerels were divided into 7 age groups: 3 days, 5 days, 7 days, 9 days, 11 days, 13 days, and 15 days after hatching. The host cockerels in all experiments were 3 days old at the beginning of the experiment.

In general, the host cockerels were kept 50 to a compartment in a battery brooder. They were allowed to feed on a stock diet of baby chick starting mash and to drink water *ad libitum* from baby chick watering founts. In most instances, the temperature was maintained at 98° F. The brooder was kept in a windowless room, illuminated constantly by artificial light. The interior of the brooder, however, was dark.

Donor cockerels were sacrificed with ether. Immediately after sacrifice, pairs of testes and adrenals were removed and frozen on dry ice for at least one hour. The gastrocnemius muscle was removed also and similarly frozen. In certain experiments adrenal and testicular tissues from adult White Leghorn roosters were employed.

A longitudinal incision was made at the base of the comb of intact 3-day host cockerels, and a pocket was hollowed out into which the grafts could be placed. Either two frozen testes or two frozen adrenal glands were implanted under the comb of the host. In the case of adult tissues, insofar as possible, a uniform amount of adrenal or testis was implanted. Precautions against infection included application of 4% gentian violet in celloidin over the incision. The control animals within each age group were of three kinds: (1) *tissue controls*, with a fragment of frozen gastrocnemius muscle engrafted under the comb; (2) *sham-operated controls*, with a longitudinal incision made at the base of the comb, followed by an application of the gentian violet-celloidin solution; (3) *reactivity controls*, with a small fragment of testosterone implanted under the comb.

All of the host cockerels were sacrificed 5 days after implantation. The body weight was recorded to 0.1 gm., and the comb excised and weighed to 0.2 mg. on a Roller-Smith balance. Comb weight/terminal body weight ratios were determined for members of all groups, and means and standard errors of the means computed.

Chicks of series B of the present study, employed in 1949–50, consisted of both male and female 3-day White Leghorns from several sources. A total of 590 were used: 82 as donors and 508 of both sexes as hosts. In addition, 3 roosters supplied certain adult tissues. Host chicks of this series received *non-frozen* transplants of 3-day chick ovary, testis, male adrenal, female adrenal, as well as adult testis and adult male adrenal. Sham-operated chicks and chicks with implants of non-gonadal tissues (primarily muscle) served as controls. In addition, other chicks (reactivity controls) received testosterone or estradiol implants. No significant difference in the response of male and female hosts was noted. The technics employed were the same as those for series A, described above,

with the exception that series B transplants were not frozen prior to implantation and, hence, can be considered as viable. The hosts of this series were not rigorously supervised in regards to temperature conditions. Although the methods employed with this series give rise to certain difficulties in interpretation of results (discussed below), the data have been summarized briefly in Table 1 for purposes of comparison with those from series A.

2. *The Castrate Rat Seminal Vesicle as a Bio-indicator of Androgenic Activity*

Sixteen male Long-Evans rats of a pure inbred strain were used. All rats were bilaterally orchidectomized when they were 6 months old and, hence, sexually mature. Five months after castration, a single midventral abdominal incision was made, and the atrophied seminal vesicles were exposed. With a Sichel knife, an incision about 1 mm. long was made in the wall of the gland. Either one frozen cockerel testis or one frozen cockerel adrenal was implanted *into the lumen* of the right seminal vesicle. A fragment of frozen cockerel gastrocnemius muscle was implanted into the lumen of the left seminal vesicle, which served as a control. The incision into the seminal vesicle was sealed with a minute patch of Gel-Foam (gelatin "gauze"). Sulfanilamide was sprinkled internally, the abdominal muscle and integument were separately stitched, and 4% gentian violet-celloidin was applied over the closed incision. The transplantation technic employed is a modification of that used by Jost (1948), who grafted fetal rat testis *under the serosa* of the seminal vesicle of adult castrate rats.

The rats were sacrificed with ether five days after operation. The seminal vesicles were removed *in toto*, fixed in Bouin's fluid, and imbedded in paraffin. Blocks were sectioned serially at 6$\mu$, and sections were stained with hematoxylin and eosin.

3. *Histochemical Methods*

Histochemical methods have been used recently in an attempt to localize ketosteroids. Albert and Leblond (1946), modifying the original technic, suggested that those areas of a particular tissue showing a yellow color with 2,4-dinitrophenylhydrazine contain plasmalogens and/or, less probably, ketosteroids. Other criticisms (*e.g.*, Vasconcelos-Frazão, 1949; Gomori, 1950) have also been directed against the use of methods such as this as evidence of ketosteroids. We do not intend to discuss the validity of the method herein.

Testes and adrenals of cockerels 3 to 15 days of age were preserved in commercial formalin which had been neutralized with $MgCO_3$. The maximum length of time the tissues remained in the preservative was 28 days and the minimum 3 days. Frozen sections were cut at 20$\mu$ and treated by the method of Albert and Leblond (1946).

A histologic study of the testes and adrenals of young cockerels was also undertaken. For these purposes, sections prepared by the Barger-Gomori technic (1947) for demonstration of alkaline phosphatase activity were utilized. A light hematoxylin stain was added to the preparations. The method is employed routinely in this laboratory and has been discussed elsewhere (Bern, 1949).

## RESULTS

### 1. *The Chick Comb as a Bio-indicator of Androgenic Activity*

Evidence that the chick comb has the ability to respond to testosterone is indicated by the size increment elicited by small fragments of the crystalline steroid implanted at the base of the comb. When compared with comb

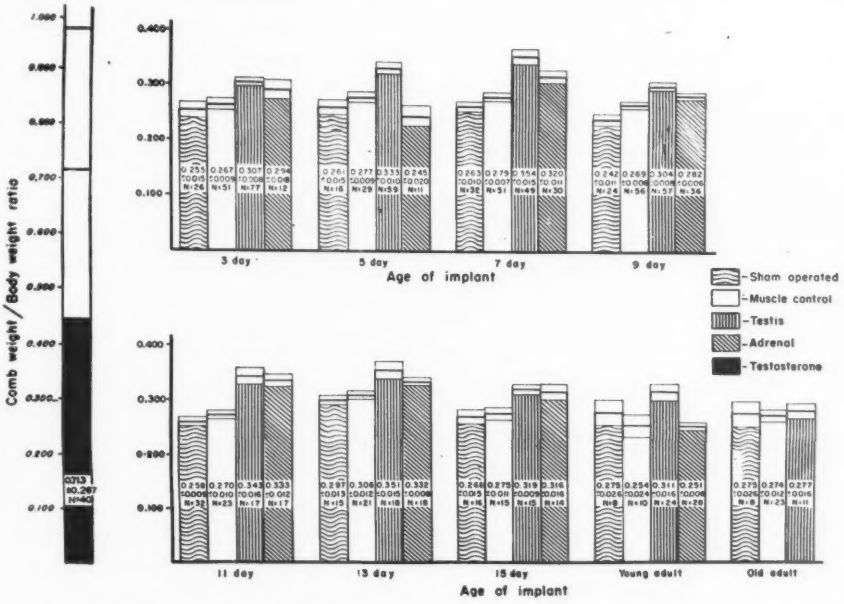


FIG. 1. Response of cockerel comb to frozen tissues implanted beneath comb. Mean is black line dividing clear space atop each block, which represents standard error of the mean.

TABLE 1. EFFECT OF IMPLANTATION UNDER THE COMB OF VARIOUS SUBSTANCES AND VIABLE (I.E., NON-FROZEN) TISSUES ON THE COMB WEIGHT/BODY WEIGHT RATIO OF CHICKS (SERIES B)

| Material Implanted | CW/BW Ratio ($M \pm S.E._m$) | Significance of Difference from Non-gonadal Controls |
|---|---|---|
| Sham-operated | $0.193 \pm 0.006$ (n = 41) | not significant |
| Non-gonadal tissues (primarily muscle) | $0.226 \pm 0.006$ (n = 81) | |
| Testosterone | $0.623 \pm 0.036$ (n = 48) | significant at 1% level |
| $\alpha$-Estradiol | $0.222 \pm 0.010$ (n = 13) | not significant |
| 3-day testis | $0.218 \pm 0.011$ (n = 35) | not significant |
| 3-day ovary | $0.221 \pm 0.007$ (n = 31) | not significant |
| 3-day male adrenal | $0.247 \pm 0.013$ (n = 20) | not significant |
| 3-day female adrenal | $0.269 \pm 0.014$ (n = 21) | significant at 1% level |
| Adult testis | $0.240 \pm 0.007$ (n = 94) | not significant |
| Adult male adrenal | $0.244 \pm 0.009$ (n = 43) | not significant |

growth observed in response to muscle transplants, the average comb weight/body weight ratio is almost three times greater after testosterone implants (fig. 1 and table 1). The variability in response was to be expected, since testosterone fragments of varying sizes were implanted.

Although there was some indication that chick muscle consistently had a non-specific stimulatory effect on comb growth as compared with sham-operated controls, the higher values obtained were not significantly different in either series A or B. However, because of the possibility of minimal non-specific stimulation, chicks with muscle implants were used as controls, and the comb growth evoked by adrenal and testis grafts compared with that occurring in response to muscle.

In series A, implants of frozen cockerel testis resulted in an increase in the comb weight/body weight ratio of the host ranging from 13% to 27%. However, there was no correlation between the age of the donor graft and per cent increase in the ratio (fig. 1). Statistical analysis of the data established that the comb weight/body weight ratios were significantly different in all groups from those after muscle transplantation. The significance was at the 1% level of confidence in all cases except the 13-day testis where the difference was significant at the 5% level.

Frozen testis fragments from a sexually mature adult rooster (about 6 months of age) implanted under the comb caused an increase in comb weight/body weight ratio of 23%, which is significant at the 1% level of confidence. Very old (non-reproductive) rooster testis, on the other hand, did not cause any significant comb increment, the ratio being only 1.1% greater than with the muscle controls.

Frozen cockerel adrenal implanted under the comb did not have a consistent effect. Grafts from 7-day and 11-day donors, when compared with muscle controls, caused increments of 15% and 24% respectively in the comb weight/body weight ratio, which are significant increases. However, adrenal grafts from the other age groups did not result in significant comb stimulation. Adult adrenal implanted under the comb elicited an insignificant decrease (1.2%) in the comb weight/body weight ratio.

In chicks of series B (table 1), receiving non-frozen implants of tissue, only the 3-day female adrenal and the testosterone implants resulted in a significant increase in the comb weight/body weight ratio. All other materials implanted evoked no significant comb hypertrophy.

In series B it was difficult to obtain large numbers of hosts with successful adrenal implants due to the occurrence of a phenomenon which we have termed "scab comb formation." Forty-four per cent of the hosts receiving 3-day male adrenal transplants, 53% of those receiving 3-day female adrenal, and 20% of those receiving adult male adrenal demonstrated this type of comb degeneration. Only two other cases of comb degeneration

were observed in this series: in one host receiving a 3-day ovary and in another receiving a 3-day testis. In some additional experiments conducted later using unfrozen 3-day adrenal, scab formation was found to occur in 29% of the host animals. In addition, this phenomenon was observed in 10 out of 76 hosts receiving 7-day and 9-day frozen cockerel adrenals (series A). No histologic examination of the degenerated comb was made, but gross observation indicated various degrees of dermal and epidermal atrophy. In some cases almost the entire comb had transformed into a "scab."

2. *The Castrate Rat Seminal Vesicle as a Bio-indicator of Androgenic Activity*

Histologic study of castrate rat seminal vesicles into which frozen cockerel testis was implanted revealed some evidence of minimal local stimulation in the area of the transplant, although no secretory granules were observed in the epithelium. In most cases the epithelium was low columnar, and glandular development seemed to be greater in the area around the transplant as compared with the castrate picture seen in those areas of the gland away from the graft. Adrenal implants likewise seemed to cause minimal epithelial stimulation. Again the epithelium was low columnar, and some glandular development was observed in the area around the transplant.

On the basis of the observations on reactions to testis and adrenal tissue alone, one might conclude that some evidence of hormonal stimulation existed. However, examination of the muscle implant controls revealed a similar picture around the graft. The changes observed, then, may be due solely to non-specific stimulation by dead tissue products and not related to the presence of steroids.

In all implants, there was extensive leucocyte infiltration, and the histologic picture of the graft was one of general necrosis. However, muscle did not completely lose its microanatomic characteristics.

3. *Histochemical Methods*

Frozen sections of cockerel testis treated with dinitrophenylhydrazine showed a slightly positive reaction regardless of age. Pale straw-colored granules were observable in the intertubular tissue. The tubules themselves, however, showed no coloration. With increasing age, up to 15 days, there was no increased intensity of stain. Sexually mature rooster testis, however, evidenced a strongly positive reaction, both in the spermatogenic epithelium and in the interstitial cells. The positive control, mouse testis, demonstrated the usual intensely colored granules in the interstitial cells. The seminiferous tubules, however, were negative, as was the negative control, mouse muscle.

Cockerel adrenal glands gave a weakly positive reaction. As with the testis, there was no differential stain intensity between the youngest (3-day) and the oldest (15-day) cockerel adrenal studied.

The histologic pictures in the testis and the adrenal were essentially the same in all the young stages of cockerel studied. In the testis, the seminiferous tubules showed a small lumen and were lined by a single layer of columnar epithelium. The stroma was composed of densely packed cells with fine cytoplasmic granules and sparse connective tissue fibers. Alkaline phosphatase activity was found throughout the intertubular stroma; none was seen in the tubules. In the adrenal, alkaline phosphatase activity was seen only in the endothelium lining the sinusoids and in the connective tissue capsule.

## DISCUSSION

Inasmuch as frozen cockerel testis at all ages studied gave evidence of stimulation of the comb (fig. 1), it seems probable that androgenic hormone is present in the testis shortly after hatching, if not before. The technics employed in this investigation, however, give no indication as to whether or not male hormone is being actively secreted at this time. That the cockerel testis contains androgen is indicated by the following observations.

1. *Implantation of testis under the comb.* Frozen cockerel testis implants from all age groups cause comb stimulation in the hosts (fig. 1). Since the implants were made directly under the comb, direct local stimulation of the target organ was possible. Any androgenic material present in the testis could diffuse out and influence the comb. However, since diffusion occurs in all directions, androgenic material not only reaches the comb but also diffuses to other surrounding tissues. Dilution of the hormone by tissue fluids undoubtedly occurs, resulting in a decreased concentration of hormone available to the comb. It seems highly probable that diffusion is the major mechanism by which androgenic hormone from the implant reaches the comb. However, some hormone may be picked up by the circulatory system.

An explanation should be made for the use of frozen implants in the definitive series (A). Release of hormone from the gland should occur more readily from tissues in which autolysis is occurring. Histologic examination of some implants revealed that cellular integrity is largely destroyed by freezing and, hence, cell permeability considerations are more or less eliminated. When transplanting living tissue, however, the success or failure of the graft is a factor to be considered in the response of target organs. If the graft is successful, a physiologic disequilibrium between the foreign tissue and the host tissue occurs. Until normal balance is restored,

normal cellular metabolism is upset. A time lag occurs while the metabolic differences between the donor and host tissues are adjusted. Hence, it is impossible to know the exact age of the transplant, since not only has an initial time lag occurred, but also successful transplants continue to grow and differentiate.

Vascular anastomoses between donor and host blood vessels or complete revascularization of the transplant must occur in the case of successful transplants. Hormone would be released primarily into the blood stream, and the possibility of direct comb stimulation would be decreased. Response elicited in the comb would result largely from hormone transported by the blood. In general, with non frozen tissues success or failure of the transplant would result in considerable variability of comb responses.

Viable transplants also would be capable of responding to host pituitary secretions, if the pituitary be functional in young cockerels. Thus, in Leroy's (1948) experiments wherein living 18-day embryonic chick testis was implanted under the comb of adult capons, there is no way of determining the age of the transplants upon termination of the experimental period. Since the transplants were successful, it is to be expected that they reacted to the high content of gonadotropins in the capon blood and, hence, secreted male hormone, regardless of whether or not they did so *in situ* (*cf.* Jost and Colonge, 1949).

A comb increment curve increasing with age of frozen testis implants was not obtained. In addition to the fact that not all of the hormone in the gland reaches the comb, it is also possible that a greater amount of hormone is not present in older cockerels, at least until the time of actual hormone secretion.

The results obtained indicate that the cockerel comb is physiologically capable of responding to androgenic hormone and that the concentration of hormone in the cockerel testis is above the threshold required for comb stimulation. No quantitative assay of hormone in the gland was made, and it is, therefore, impossible to know the amount of male hormone present. Dorfman (1948) has shown that the comb of White Leghorn chicks responds to 2 micrograms of testosterone propionate. It seems reasonable to conclude that within 3 days after hatching cockerel testis contains at least a comparable amount of male hormone.

2. *Implantation into the seminal vesicle.* Jost (1948) and Jost and Colonge (1949) transplanted living 15- to 16-day fetal rat testis under the serosa of the seminal vesicle of adult castrate or castrate and hypophysectomized rats, and noted evidence of intensive localized androgenic activity, which was not observed with control tissues such as kidney. Successful transplants were capable of responding to physiologic factors within the body of the host. Interstitial cell hypertrophy of the transplant was noted in

the rats which were castrated but not hypophysectomized. The observed hypertrophy undoubtedly was a response to the high titer of gonadotropins present in castrate rat blood. On the other hand, no interstitial cell development occurred in the grafts made into hosts which were both orchidectomized and hypophysectomized. However, Jost and Colonge (1949) pointed out that in both castrate and castrate-hypophysectomized hosts, the seminal vesicle epithelium showed evidence of response to androgenic hormone, though quite limited in the latter.

On the basis of our observations on the reactions to implants of frozen cockerel testis, adrenal, and muscle, it seems that the seminal vesicle epithelium of castrate rats responds minimally to non-specific stimulation by foreign tissue. Since these transplants were made directly into the lumen of the gland there was no problem of dilution by tissue fluids. Nevertheless, the reaction observed was very slight compared with that pictured by Jost (1951).

3. *Ketosteroid determination.* There is considerable evidence that the dinitrophenylhydrazine reaction does not localize ketosteroids (Albert and Leblond, 1946; Vasconcelos-Frazão, 1949; Gomori, 1950). However, immature testis was only slightly positive, whereas sexually mature adult rooster testis and mouse testis were strongly positive. It is established that sexually mature roosters and mice are actively secreting male hormone, whereas in the immature cockerel there is evidence of hormone production but not necessarily of hormone release. The dinitrophenylhydrazine reaction may indicate a metabolic difference between the adult and immature testis, such as in "plasmalogens," even if ketosteroids themselves are not localized. We have no explanation to offer for the positive reaction of the spermatogenic epithelium in the adult rooster.

In summary, the evidence from testis implants under the comb of 3-day cockerels seems to indicate that androgen is being produced in the testis of immature cockerels. The observations of castrate rat seminal vesicle stimulation and of ketosteroid localization do not oppose this conclusion. It is thus possible for secretion to occur as early as 3 days after hatching, if not before, if other factors (hypophyseal) are operative.

In regard to the possible androgenic activity of the adrenal, except for two groups, the implants under the comb caused no significant comb increment. Therefore, there is some doubt relative to the testoid content of the immature cockerel adrenal. Likewise no conclusive statements can be made relative to the effect of adrenal implants into the seminal vesicles of castrate rats or to the significance of the dinitrophenylhydrazine reaction.

The frequent comb degeneration observed after adrenal implantation is of some interest. It has been demonstrated that adrenal corticosteroids cause epidermal and dermal degeneration in mammals (Baker and Whit-

aker, 1948; Castor and Baker, 1950). In considering the "scab comb" effect as a manifestation of dermal and epidermal atrophy, we suggest that the degenerative changes may have been initiated by C-11 oxygenated steroids present in the maturing and mature adrenal.

## SUMMARY AND CONCLUSIONS

1. Testis and adrenal of young White Leghorn cockerels (3–15 days after hatching) and adult roosters were examined for evidence of androgen production by:

a. Implantation of frozen tissue at the base of the comb of 3-day White Leghorn cockerels;
b. Implantation of frozen tissue into the lumen of the seminal vesicle of castrate male rats;
c. Localization of ketosteroids or associated substances by the dinitrophenylhydrazine reaction.

2. Implantation under the comb indicates that the testis contains androgen. The other methods employed are inconclusive in establishing this point, but results are not in conflict with this conclusion. Testis *production* of hormone as opposed to *secretion* is emphasized.

3. Results from studies of possible adrenal testoid activity are inconclusive. "Scab" formation in the comb was noted in approximately 40% of host chicks with non-frozen 3-day adrenal transplants, in 20% of hosts with non-frozen adult adrenal, and also in some hosts with frozen 7-day and 9-day adrenal transplants. It is suggested that this degenerative phenomenon may be related to the presence of C-11 oxygenated corticoids.

4. The advantages of using frozen tissue, and also the desirability of using non-endocrine tissue as a control in implant work, are emphasized. Non-specific stimulation, the role of the host pituitary gonadotropin factor, and variability owing to success or failure of transplants were largely controlled in the present studies.

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# DIFFERENTIAL REACTIVITY OF INDIVIDUALS AND THE RESPONSE OF THE MALE GUINEA PIG TO TESTOSTERONE PROPIONATE[1]

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MANY investigators have directed attention to the close relationship between the reactivity of tissues, sometimes referred to as sensitivity or responsiveness, and the degree of response to hormonal stimulation. The literature in which the subject is discussed has never been reviewed completely, but a few references will indicate something of the extent to which differences in reactivity influence endocrine function.

Smith and Engle (1927) reported that the response of immature rats and mice to pituitary transplants increased with age; that in older animals fewer transplants were required to produce precocious sexual maturity. Bradbury (1944) and McCormack and Elden (1945) found an inherent seasonal variation in the sensitivity of the rabbit to pituitary extracts. Albright, Burnett, Smith, and Parson (1942) presented evidence that in certain clinical cases of idiopathic hypoparathyroidism the disturbance was not a lack of hormone, rather a resistance to it. Selye and Albert (1942) described a differential reactivity of the adrenal gland. They found that estradiol administered to immature male rats in large doses was not followed by the adrenal cortical hypertrophy that was produced in the adult, in fact, it tended to decrease the size of the tissue. Sprague (1951) writes that in many conditions of seeming adrenal insufficiency, no deficiency of the adrenal hormones exists. Apparently, there had been an alteration in the manner in which the target organs were responding to the hormones.

The importance of the relationship of reactivity for tissue response is not restricted to mammals. Lillie (1932) showed that different feather tracts of the brown leghorn fowl have inherently different growth rates, and exhibit correspondingly different thresholds of reaction to the female hormone. The response of the oviducts in young New Hampshire Red chicks to injections of stilbestrol was shown to depend on the level of folic

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acid intake (Hertz, 1945). Williams (1942), early in his work on the metamorphosis of the native silkworm moth, concluded that the ultimate result is determined by the ability of the tissues to react.

Early reports of differences in the reactivity to gonadal hormones and an enumeration of some of the factors which might influence reactivity have been reviewed (Young, 1941; Beach, 1948), but there are more recent additional studies. The seminal vesicles of the rat are most sensitive to stimulation by androgens at 40 to 60 days of age, a time when this tissue is differentiating most rapidly (Hooker, 1942). Price (1944) indicated that at 26 days there is a marked increase in the reactivity of the uterus and seminal vesicle of gonadectomized rats to testosterone propionate. A similar change was found in the male and female prostate on the thirty-sixth day. Hamilton (1948) found that no correlation exists between certain secondary sexual characteristics of the human male, such as baldness and the presence of auricular hair, and the titers of excreted steroids. He concluded that the action of the hormones in endocrine-dependent states is controlled by genetic, aging, environmental, and other factors. Lyman and Dempsey (1951) working with hibernating, castrated, male hamsters showed that following the injection of testosterone, the seminal vesicles of the animals which returned to hibernation revealed little enlargement or histological change, whereas those of the animals which remained awake showed an increase in weight and histological alteration. They concluded that it is not the amount of circulating hormone which determines the condition of the affected organ, rather the transient condition of the organ.

It will be apparent that most of these differences in reactivity have been associated with age, season, and nutrition. Other factors however may also be involved. Regulation of the reactivity of tissues by endocrine substances, such as the thyroid hormone, has been suggested (Smelser, 1939; Salmon, 1941; Langham and Gustavson, 1947; and others). Another example is the priming effect of an initial dose of estrogen on the sensitivity of the vaginal mucosa (Kahnt and Doisy, 1928). Differences in reactivity may have a genetic basis. Bates, Riddle, and Lahr (1941) report that two hereditary strains of chicks showed differential thyroid responsiveness to thyrotrophin. Chicks from one source required four times as much hormone to produce an amount of stimulation equal to that obtained in chicks from the other.

In few if any of the studies cited above were the investigators concerned primarily with reactivity. Most information came as a by-product of experiments directed toward other problems. Lacking was a study of the extent and importance of differences in the reactivity of tissues for hormonal action in animals homogeneous with respect to age, diet, the season of experimentation, methods of handling, and general endocrine balance

as judged by the condition of the individuals at the beginning of the investigation.

## MATERIALS AND METHODS

Reproductive behavior of the male is particularly well adapted for investigations of this type. The end points are definite. The strength of sex drive displayed by individual male guinea pigs is extremely variable from animal to animal, but for individuals quite constant from test to test. Long range experiments requiring comparatively few animals are possible. Individual animals can be used repeatedly giving continuous information throughout the experimental period with a minimum of operational trauma. The importance of this last point can be illustrated briefly. In this investigation each animal was given 41 tests, consequently much more information was obtained from single animals than would have been possible had procedures been employed that involved sacrifice of the animals following each test.

Sexual behavior patterns of the adult, male guinea pig and their development have been described (Avery, 1925; Louttit, 1927, 1929; Sollenberger and Hamilton, 1939; Seward, 1940; Young and Grunt, 1951; and Webster and Young, 1951), but since an understanding of what follows depends on some familiarity with the behavior, a brief description is given.

When a female guinea pig in heat is placed with a male the following sequence of behavior is usually seen. The male follows the female and there is frequently a generalized sniffing and licking, or pulling of the hair with the teeth. The term *nibbling* is used to describe these actions. Shortly afterwards, the male centers his attention on the anogenital region; *nuzzling* is used for this localized sniffing and licking. In the next type of action the male mounts the female and executes a series of pelvic movements. Most often this occurs posteriorly; frequently, however, in his excitement the male mounts elsewhere. In either case, the action is called *mounting*. In the display of the complete pattern terminating in ejaculation, *intromission* with pelvic thrusts usually follows mounting. In those cases in which ejaculation occurs, the activity is brought to an abrupt end with a grasping of the female's back, and a convulsive drawing in of the flanks. This position is held for a number of seconds following which the male dismounts and licks his penis. If watched, he will be seen to drag his pudendum along the floor of the cage. Usually the time required to achieve ejaculation is less than 10 minutes. More frequently than not, ejaculation is followed by a period of quiescence which lasts at least an hour (Grunt and Young, 1952). This sequence of behavior is not invariable. At times the lower degrees of the behavior pattern are omitted and mounting with intromission or even ejaculation takes place almost immediately. At other times there is little sexual behavior beyond nuzzling or mounting.

Although there is wide variation in the age at which these elements of the sexual behavior pattern are first displayed, the approximate age at which each component appears is known. Generally, nibbling is first seen about the fourteenth day, nuzzling about the seventeenth day, mounting about the forty-fifth day, intromission about the fifty-fourth day, and ejaculation about the sixty-fourth day (Avery, 1925; Louttit, 1929; and Webster and Young, 1951). All this information was utilized in the development of the method (Young and Grunt, 1951) by which the strength of sex drive was measured in this investigation.

When determining the strength of sex drive a female displaying good heat responses (Young, Dempsey, Hagquist, and Boling, 1937) was placed in the cage occupied by a male. The amounts of the five measures of behavior, nibbling, nuzzling, mounting, intro-

mission and ejaculation, displayed by the male during a 10-minute period were recorded. Each minute of the test was divided into four 15-second periods, and a measure was recorded only once during each period in which it was shown. Intromission and ejaculation, being more discrete and of shorter duration, were recorded only in the period during which the measure of behavior began.

In determining the score for each test, the number of 15-second periods was counted during which each measure of behavior was displayed. Only the highest measure of behavior displayed during each period was used in the calculations. Therefore, the maximum number of measures in each test was 40. Since, however, ejaculation usually is achieved in less than 10 minutes, the number of measures was less. The number of 15-second periods during which a given measure of behavior was displayed was then divided by the duration of the test. The quotient represents the number of times per minute when that measure of behavior was the highest degree shown. Each quotient was then multiplied by a factor arbitrarily chosen as representing the importance of the measure; 1 for nibbling, 2 for nuzzling, 3 for mounting, 4 for intromission, and 5 for ejaculation. These values were chosen because, as was indicated above, the elements of the behavior pattern appear in this order during maturation. The sum of the products was taken as the score of the test. When this procedure is followed, an animal that mates within the first 15 seconds is scored 20.0 while one that does nothing for the entire 10 minutes of the test is scored 0.0. A male having an average of 8.50 or higher for 10 tests is considered *high drive*. If the score averages between 6.25 and 8.50, he is considered *medium drive;* if below 6.25, *low drive*.

In the first of two experiments, each of 39 male guinea pigs, 80 to 150 days of age, was isolated in a cage 24 inches square. Beginning approximately seven days later each male was given tests at weekly intervals to determine the strength of his sex drive. After 10 tests all but 10 of the animals, the intact controls, were castrated. Their average scores ranged from 2.2 to 11.1. For the following 31 weeks both experimental and control animals were tested weekly. In all 1599 tests were made. During the first 16 of the 31 weeks no therapy was given. This was the period during which sexual activity decreased from the precastrational level to a constant low level, which is referred to as the *base line*. During the following 15 weeks, each of 22 of the castrated males was injected with testosterone propionate.[3] The androgen was administered daily in doses of 25γ per 100 grams body weight for the first 10 weeks of this period, and 50γ per 100 grams body weight for the last 5 weeks. The volume of injected sesame oil containing dissolved hormone was kept constant for each 100 grams body weight. Seven of the 29 castrates were given sesame oil and served as the castrate control group.

At the end of the experiment the scores for each week were averaged. In a first analysis of the scores the animals were divided into intact controls, castrate controls and castrates given testosterone propionate, thus revealing the effects of castration and subsequent androgen therapy. A second type of analysis was suggested by the great differences between individuals. For this analysis the animals were divided into high, medium, and low drive groups, thus relating the performance of each group during the period of therapy to the strength of drive prior to castration.

In the second experiment 31 animals were used. On the basis of the scores of 10 tests prior to castration, these males were divided into four balanced groups whose scores averaged 7.8. Each group contained 7 or 8 animals. Following castration, tests were made at weekly intervals for 31 weeks. During the first 16 weeks no androgen was given,

[3] Testosterone propionate was supplied by the courtesy of Ciba Pharmaceutical Products, Inc.

and the scores decreased to the base line. At the beginning of the seventeenth week following castration and for the remaining 15 weeks, the animals in each of the four groups were given daily injections of testosterone propionate in doses of 12.5, 25, 50, or 100γ per 100 grams body weight. These amounts were used in order that the effects of a variable dosage could be compared. In all, 1271 tests were made.

Following the last test, the weekly scores were averaged. In a first analysis, the averages for each of the four groups were plotted in order to show the effects of castration and therapy with these amounts of androgen. In a second analysis the performance was related to the strength of drive prior to castration.

After the last test, the animals were sacrificed and portions of the endocrine and reproductive systems were fixed for histochemical and histological study. The results of the microscopic findings will be reported elsewhere.

Throughout the work the temperature was maintained between 70° and 75° F. The diet always included rabbit pellets, oats, green vegetables, alfalfa hay, and water. All the animals were healthy, and showed little if any effect of the operation or daily injections.

## RESULTS

The results obtained from the experiment involving observation of males prior to castration, during the period of castration without therapy, and while testosterone propionate was being administered in the same amount per 100 grams body weight to each animal are summarized in figure 1.

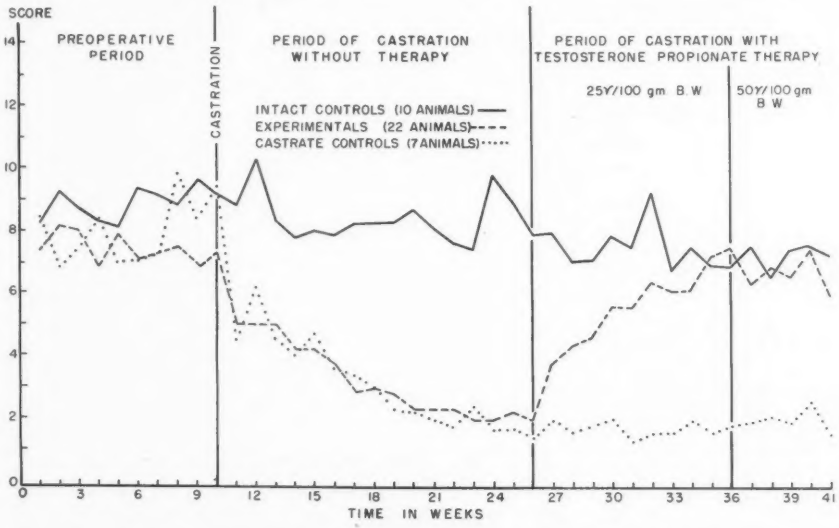


FIG. 1. Strength of sex drive in male guinea pigs before and after castration and following therapy with testosterone propionate daily.

During the 41 weeks of the experiment the intact controls showed a very slow decline in sexual activity. By the end of the period approxi-

mately 18% of the original drive had been lost. This loss manifested itself mainly in a decline in the frequency of ejaculations which was possibly associated with aging. Removal of the gonads in the animals to be studied experimentally was followed by a gradual decline in sexual activity. The base line was reached within 14 weeks, during which time more than 75% of their original drive was lost. The activity at this level consisted basically of nibbling and the score achieved was approximately 2.0. The activity of the animals which received no treatment save the daily injection of sesame oil remained at this level during the remainder of the experiment. When the experimental animals were given testosterone propionate beginning

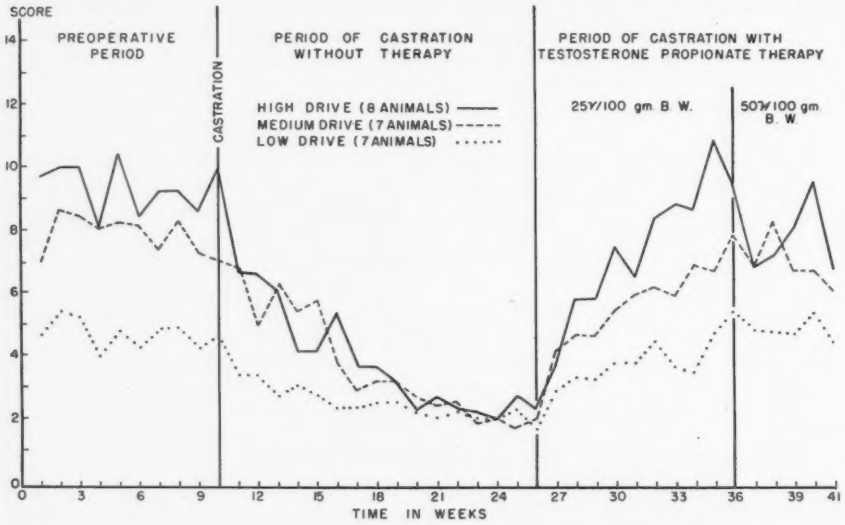


FIG. 2. The effect of castration and therapy with testosterone propionate daily on the sex drive of high, medium, and low drive male guinea pigs.

the seventeenth week following castration, their sexual activity increased. By the tenth week after the start of therapy, they returned to a level of drive similar to that of the intact controls, and only slightly lower than that seen prior to castration.

It will be recalled that the 22 castrated animals given testosterone propionate were divided into three groups according to the strength of their sex drive prior to castration. The average scores of these groups before castration, during the period of castration without therapy, and after androgen treatment was begun are shown in figure 2. Almost immediately after the beginning of testosterone propionate therapy a redistribution of the animals into the groups seen prior to castration became apparent. The

high drive animals showed the highest amount of sexual activity, the low drive animals the lowest, and in general, the activity of the medium drive animals was between the two. Not once during the period of androgen therapy did the score of the low drive group equal that of the medium or high drive groups. In all three groups the strength of drive following androgen administration was comparable with that prior to castration.

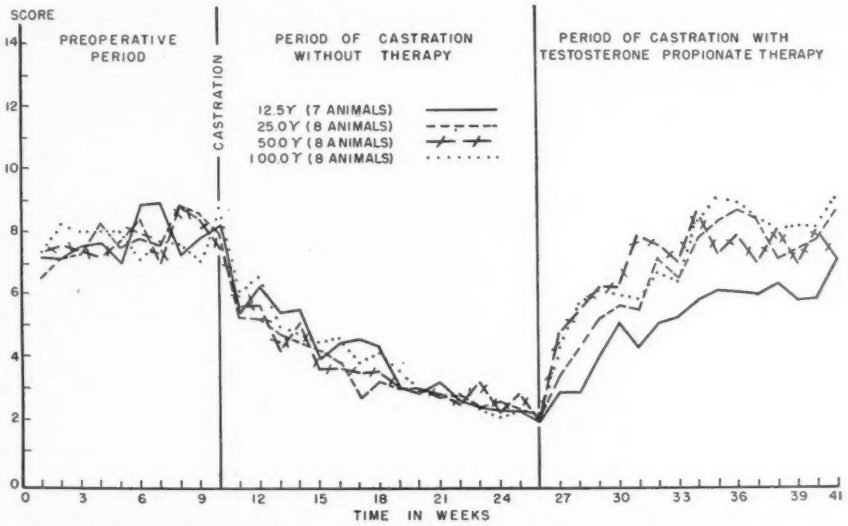


FIG. 3. Strength of sex drive in male guinea pigs before and after castration and following therapy with different quantities of testosterone propionate daily.

The significance of the differences between the groups was tested by the "t" test. In doing so the scores of the tests for the thirty-fourth, thirty-fifth, and thirty-sixth weeks were used. This was the end of the period during which therapy consisted of 25γ testosterone propionate per 100 grams body weight. It was found that the differences between the low drive group and the other two were significant at the 2% level or better. Because of the variability within the medium drive group, the difference between this group and the high drive group was not significant, nevertheless, the trend was apparent. Similar comparisons were made for the thirty-ninth, fortieth, and forty-first tests. This was the end of the period during which therapy consisted of 50γ testosterone propionate per 100 grams body weight. Although the same trend manifests itself, the increase in variability was sufficient to decrease the significance of the differences. Only that between the high and low drive animals remained significant, but at the

3% level. The correlations of the data from all the castrates given androgen, when compared with the average of the precastrational scores, were found to be significant at the 1% level or better.

With few exceptions, the results of the experiment in which four different doses of androgen were administered were comparable with those obtained when each animal received the same amount (figure 3). As before, sexual drive of the four groups decreased to the base line after castration and, following the injection of testosterone propionate, there was an increase in sexual behavior. The only conspicuous deviation from the rule

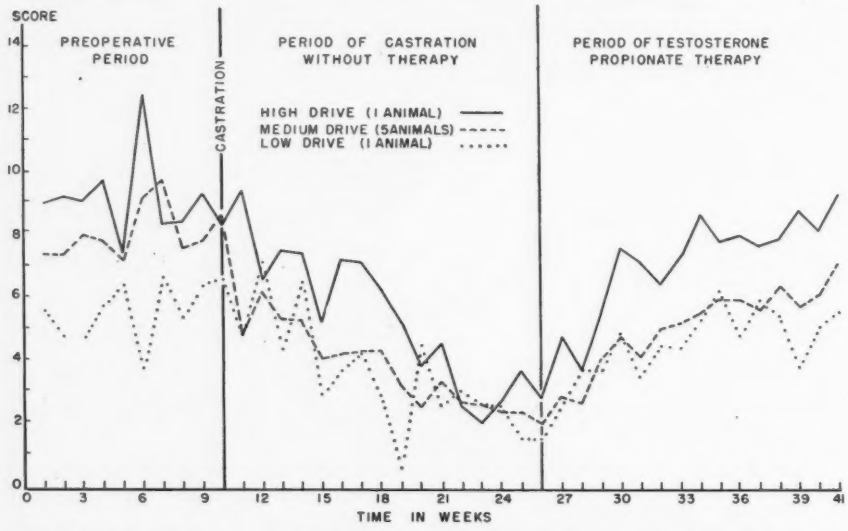


FIG. 4. The effect of castration and therapy with 12.5γ testosterone propionate daily on the sex drive of high, medium, and low drive male guinea pigs.

that individuals return to the level displayed prior to castration, was that the rate of increase in the sexual activity of the group given daily injections of 12.5γ testosterone propionate per 100 grams body weight was slower than in the other animals. Never during the 15 weeks of therapy did this group reach its precastrational average, although at the end of the experiment there were indications of a return to this level. The low scores were due basically to the failure to achieve intromission and ejaculation.

By the eighth week after the beginning of testosterone propionate therapy, the sexual drive of the animals in the other three groups had returned to the precastration level. With minor exceptions, they continued to dis-

play sexual activity of this degree for the remainder of the experiment. The behavior of the animals given 100γ testosterone propionate was comparable with that of the others, except that, starting 9 weeks after the beginning of therapy, the scores always equalled or surpassed by a slight degree, the highest scores of the other groups. The differences were tested for significance. Using the means of the last five tests, it was found that only the differences between the group given 12.5γ testosterone propionate and each of the other groups were significant at the 5% level. None of the other

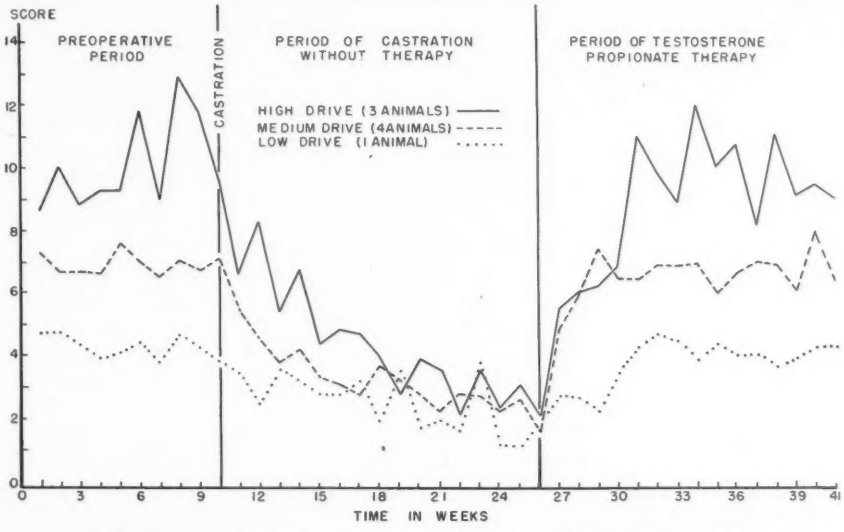


FIG. 5. The effect of castration and therapy with 50.0γ testosterone propionate daily on the sex drive of high, medium, and low drive male guinea pigs.

differences was significant. It is seen, therefore, that once the threshold was reached, 4-fold differences in dosage did not cause significant variation in sexual behavior.

In the first part of the experiment it was shown that when male guinea pigs are injected with a given dosage of androgen, the high drive animals returned to a high level of activity, and the low drive animals returned to a low level. It became of interest therefore, to see whether this would also be true when the range of experimental dosages was varied as in the second experiment. Even though the number of animals in each group was less, comparable data were obtained (figures 4, 5, and 6). The strength of drive following castration and androgen therapy was related to that prior to castration. The response of the eight animals receiving 25γ testosterone

propionate are not summarized here because it was similar to that of the animals used in the first part of the experiment (figure 2).

## DISCUSSION

Although the importance of the reactivity of a tissue or organ for its responsiveness to hormonal stimulation has been widely noted, this study is believed to be one of the first, if not the first, in which adult animals, homogeneous with respect to age, diet, the season of experimentation, method of handling and caging, have been used. The degree of variation in the hormonally induced reaction that was studied is of interest. More so,

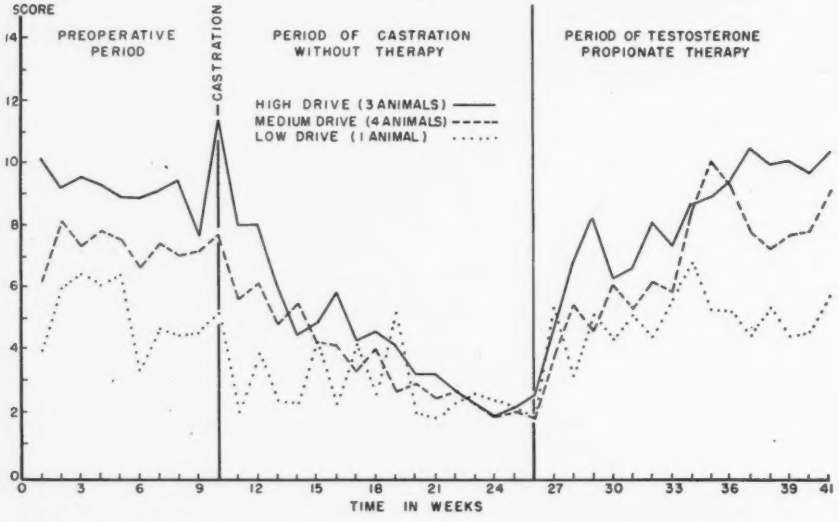


FIG. 6. The effect of castration and therapy with 100γ testosterone propionate daily on the sex drive of high, medium, and low drive male guinea pigs.

however, is the demonstration of the faithfulness with which the strength of the precastrational sex drive of individuals was restored by hormonal treatment following castration, and, in the second part of the experiment, the demonstration that within the limits of a 4- to 8-fold increase above the threshold, variation in strength of drive was attributable to the reactivity of the tissues on which the hormone was acting rather than to variation in the amount of hormone. Increased emphasis is thereby given to the principle that the response to a hormone is influenced by the character of the soma.

The reader will want to know whether this suggestion is consistent with other data that are reported by those who have followed the effects of

androgens on adults. Hamilton's (1948) demonstration that testosterone propionate will not cause alopecia in some eunuchs, particularly those having relatives that are not bald, is similar in principle. The point was also made by Dempsey (1951) when he called attention to the observation that certain patients with interstitial cell tumors, although producing great amounts of androgen, fail to exhibit any of the symptoms usually associated with high levels of testosterone. The results from two other studies, on the other hand, indicate that the response may be proportional to the amount of hormone. Gordon and Fields (1942) showed that large amounts of androgen, when given to hypogonadal individuals often cause excessive penis growth, priapism, and "over strong sex interest." Beach and Holz-Tucker (1948) indicated that castrated male rats receiving 100 to 500γ testosterone propionate per day exhibited sexual behavior that was equal or superior to that shown prior to gonadectomy. Species differences may account for the lack of agreement, but even this suggestion is premature because no two studies are more than remotely comparable. Additional work must be done before the principle that is postulated can be fully evaluated. Also of importance is the full meaning of reactivity in terms of what is taking place within the cells that are being stimulated by hormones. No approach to the problem was made during this study, nor is any suggested in the literature.

## SUMMARY

The strength of sex drive displayed by individual male guinea pigs is extremely variable from animal to animal, consequently the tissues mediating this behavior are well adapted for a study of the relationship between tissue reactivity and the response to testosterone propionate. Male guinea pigs, homogeneous with respect to age, diet, the season of experimentation, methods of handling, and general endocrine balance as judged by the conditions of the individuals at the beginning of the investigation, were used. The administration of 12.5, 25, 50 or 100γ of the androgen per 100 grams body weight daily led to the demonstration that the strength of sex drive displayed by individuals during therapy is similar to that shown prior to castration. With androgen therapy, animals that were characteristically high drive prior to gonadectomy returned to the high level, while the animals that were low drive returned to the low level. It is postulated from the results obtained when the tissues mediating sexual behavior in the male guinea pig were used as the target organ, that much of the difference between individuals is attributable to the reactivity of the tissues rather than to differences in the amount of hormone. The strength of the reaction to the androgen was not altered significantly by a 4- to 8-fold increase in dosage.

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# INCREASE IN THE THYROTROPIC HORMONE CONTENT OF BLOOD AFTER THYROIDECTOMY AS SHOWN BY PARABIOSIS[1]

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THIS study shows that a greater content of thyrotropic hormone in blood after thyroidectomy can be demonstrated by parabiosis of a thyroidectomized rat with an hypophysectomized partner. The cross-transfusion technique of parabiosis has been shown to be an excellent technique for demonstration of the reciprocal regulatory control between the pituitary and its target organs. The increase in follicle-stimulating hormone in the circulation following castration has been demonstrated by parabiosis of a normal female with a castrated partner (Matsuyama, 1921). The increased adrenocorticotropic hormone in the circulation following adrenalectomy has been demonstrated by uniting adrenalectomized rats with hypophysectomized partners (Jacobsohn, 1949, and Van Dyke, Simpson, Li and Evans, 1950).

Several unsuccessful attempts have been made to demonstrate a high thyrotropic hormone content of blood after thyroidectomy by establishing a parabiotic union between thyroidectomized and normal rats (Zayko, Ikonen, 1934, Zeckwer, 1940, Griesbach, Purves, 1943, and Griesbach, Kennedy, 1949), yet an increased thyrotropic activity in the serum of thyroidectomized rats was demonstrated by Griesbach and Purves when the serum was tested in appropriately prepared guinea pigs (Griesbach and Purves, 1943). The increase in the circulating thyrotropic hormone could probably be demonstrated parabiotically by the union of a thyroidectomized rat with a more sensitive test animal, an hypophysectomized partner. The present study employs such a combination.

## MATERIALS AND METHODS

Littermate male rats[2] were parabiotically united at 28 days of age by the open coelomic method of Paul Bert (1864). On the 75th day one member was either thyroidecto-

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[1] Aided by a grant from the United States Public Health Service, GG 409 (C3).

[2] Highly inbred rats (brother-sister inbreeding for more than 50 generations) of the Slonaker strain were used in this experiment.

mized or hypophysectomized to establish the two important combinations of the experiment:

(1) Thyroidectomized rats with hypophysectomized partners (T-H). (2) Normal rats with hypophysectomized partners (N-H). The following combinations were prepared to serve as controls: Normal with normal (N-N), thyroidectomized with thyroidectomized (T-T), hypophysectomized with hypophysectomized (H-H), and normal with thyroidectomized (N-T). All animals were maintained on a complete diet.[3] Autopsies were performed 30 days post-operatively at the age of 105 days.

The thyroids were fixed in Bouin's fluid, weighed, and were sectioned at 6 micra parallel to the longest diameter. Medial sections were selected and stained with iron hematoxylin-aniline blue (Koneff, 1936). The average height of the thyroid epithelium was determined for each thyroid by measuring 100 camera lucid tracings of follicular epithelial cells. Only those cells which fell along a line through the longest diameter of the section were measured.

The pituitaries were fixed in Zenker-formol, weighed, embedded in nitrocellulose, cut at 4 micra and stained with modified Mallory-Azan stain (Koneff, 1938). Not less than 2500 cells were counted from each anterior lobe to determine the percentage of different cell types.

The adrenals were weighed and treated according to Flexner and Grollman's (1939) method for the demonstration of lipid distribution. The size of the left adrenal cortex in each animal was determined by measuring (with the planimeter) the projected outlines of four medial, but not consecutive sections. From these measurements the group averages were calculated and expressed in planimeter units.

The completeness of hypophysectomy was verified under a dissecting microscope at the time of autopsy. No incomplete hypophysectomies were included. The completeness of the thyroidectomy was checked in serial sections of the thyroid region. In order to have a definite concept of the amount of thyroid left in the instances of incomplete thyroidectomy the following procedure was employed: Medial sections of all normal thyroids were projected on paper and the area measured with the planimeter. In the same manner the area of the thyroid tissue fragment (the largest found in serial sections) was determined.

The thyroids had been completely removed from 30 per cent of the rats subjected to thyroidectomy. The remaining animals had fragments representing from 0.7 to 8.0 per cent of the total thyroid tissue. This should be kept in mind since, for the sake of simplifying an already complicated reference system, all animals in which thyroid ablation was attempted are referred to in this paper as thyroidectomized. It also should be remembered that to demonstrate the reciprocal control between thyroid and pituitary requires thyroxine deficiency but not necessarily absence of thyroxin.

## RESULTS

The comparison of the weights of thyroids of hypophysectomized rats receiving blood from *normal* partners with those receiving blood from *thyroidectomized* partners is the basis of this study. The thyroids of the

[3] Diet I (modified from McCollum's formula consists of 67.5% whole wheat, 15% casein, 10% whole milk powder, 0.75% NaCl, 1.5% $CaCO_3$, 5.25% hydrogenated vegetable oil, and a concentrate of fish oil in amount to give 19 U.S.P. units of Vitamin A and 2.5 A.O.A.C. chick units of Vitamin D per gram of diet.

hypophysectomized members united with normal partners were stimulated only slightly and weighed 8.9 mg. as compared to the thyroids of the hypophysectomized controls united with thyroidectomized partners which weighed 7.2 mg. There was also a small but definite increase in follicular cell height over that of hypophysectomized pairs (H-H), table 1 and plate 1, figs. 2 and 3. The thyroids of hypophysectomized rats parabiosed with thyroidectomized partners (H-T) were equal to the normal controls both in weight and height of follicular epithelium (table 1 and plate 1, figs. 1 and 5).

TABLE 1. AVERAGE BODY, PITUITARY AND THYROID WEIGHTS AND CELL HEIGHT OF THE THYROID EPITHELIUM IN THE FIRST-MENTIONED MEMBERS OF PARABIOTIC PARTNERS*

| Type of pair | No. of pairs | Average body weight at autopsy | Average pituitary weight | Thyroid | | Cell height compared SR‡ | |
|---|---|---|---|---|---|---|---|
| | | | | Average weight | Average cell height | | |
| | | gm. | mg. | mg. | (μ) | | |
| N-N | 6 | 156 | 6.02 ± .19† | 14.80 ± .47 | 10.38 ± .44 | H-N vs. H-H | 6.48 |
| T-T | 6 | 138 | 8.87 ± .39 | — | — | N-T vs. N-N | 5.02 |
| H-H | 6 | 96 | — | 7.19 ± .15 | 3.55 ± .16 | H-T vs. N-N | 1.89 |
| N-H | 7 | 162 | 5.86 ± .23 | 14.57 ± .76 | 12.42 ± .33 | | |
| N-T | 7 | 157 | 6.34 ± .23 | 14.66 ± .65 | 12.94 ± .27 | | |
| T-N | 7 | 162 | 7.66 ± .41 | — | — | | |
| T-H | 7 | 173 | 7.03 ± .45 | — | — | | |
| H-N | 7 | 135 | — | 8.91 ± .46 | 5.30 ± .20 | | |
| H-T | 7 | 129 | — | 14.58 ± 1.10 | 11.78 ± .61 | | |

* N = normal, T = thyroidectomized, H = hypophysectomized.

† SE (of mean) = $\frac{SD}{\sqrt{n-1}}$.

‡ SR = Significance ratio. The difference of the means was considered statistically significant if the SR was greater than 3.0.

The epithelial cells of the thyroids of normal partners in N-T combinations were higher than in the N-N controls (table 1 and plate 1, figs. 1 and 4). On this basis and also on the basis of more numerous colloid droplets in the cytoplasm, these thyroids were considered more active than those of the (N-N) controls.

The pituitaries of thyroidectomized controls (T-T) were heavier than normal (N-N) (table 1) and showed the well known thyroidectomy changes involving both of the chromophil cells (plate 2, fig. 2). The acidophils were degranulated and the number of these cells was consistently reduced from the normal of 39.44% to 1.07 %(table 2). The percentage of basophils was significantly increased, from 5.35% to 13.65%. The basophils showed multiple vacuolation of the cytoplasm, enlarged Golgi apparatus and prominent nucleoli.

The pituitaries of thyroidectomized animals united with normal partners (T-N) showed normal percentages of acidophils but an increase in the number of basophils (table 2, plate 2, fig. 3). The partial nature of the

thyroidectomy changes indicates that some thyroxin deficiency still existed in these rats prior to autopsy. The same condition existed in the pituitaries of the thyroidectomized members of the T-H pairs. They were slightly heavier than normal and showed typical thyroidectomy changes in many basophils (plate 2, fig. 4).

Of all other organs examined only the adrenals showed changes of interest. The adrenals of hypophysectomized parabionts (H-H) were atrophic as would be expected (table 3). The union of hypophysectomized rats with a partner possessing a pituitary either normal or thyroidectomized (H-N or H-T) resulted in partial maintenance of the adrenal. All cortical zones of these adrenals were wider than in hypophysectomized controls and there was more lipid, especially in the zona glomerulosa. The most signifi-

TABLE 2. PERCENTAGE OF CELL TYPES IN THE PITUITARIES* OF PARABIOTIC PARTNERS

| Type of pair | Pituitary cells (%) | | | Percentage of cells compared SR‡ | | |
|---|---|---|---|---|---|---|
| | Acidophils | Basophils | Chromophobes | | Acidophils | Basophils |
| N-N | 39.44 ± .82† | 5.35 ± .13 | 55.21 ± .80 | T-T vs. N-N | 46.80 | 5.03 |
| T-T | 1.07 ± .07 | 13.63 ± 1.64 | 85.30 ± 1.65 | T-N vs. N-N | 1.09 | 7.07 |
| T-H | 38.32 ± 2.22 | 8.05 ± 1.06 | 53.63 ± 2.09 | T-H vs. N-N | 0.47 | 2.52 |
| T-N | 38.35 ± .60 | 11.29 ± .83 | 50.36 ± 1.24 | | | |
| N-T | 42.95 ± 1.25 | 5.35 ± .58 | 51.70 ± 1.49 | | | |

* Six pituitaries from each group studied.

† SE (of mean) $= \frac{SD}{\sqrt{n-1}}$

‡ SR = Significance ratio. The difference of the means was considered statistically significant if the SR was greater than 2.0.

cant change in the adrenals was seen in the thyroidectomized members of thyroidectomized-normal pairs. These adrenals showed a significant increase in size of cortex and in weight over those of the normal controls (N-N).

## PLATE I

Photographs were taken with 8 mm. objective at magnification ×250 (reduced 1/6). Iron hematoxylin-aniline blue stain.

FIG. 1. Thyroid gland from a normal control rat in N-N pair.

FIG. 2. Thyroid gland from a hypophysectomized rat in H-H pair.

FIG. 3. Thyroid gland from a hypophysectomized partner in H-N pair. Note the better condition of nuclei and cytoplasm of the thyroid epithelium than that shown in fig. 2.

FIG. 4. Thyroid gland from a normal partner in N-T pair. Note the greater than normal activity shown by nuclei, epithelial height and follicular colloid. Compare with fig. 1.

FIG. 5. Thyroid gland from a hypophysectomized partner in H-T pair. The gland has been maintained in normal or better than normal activity.

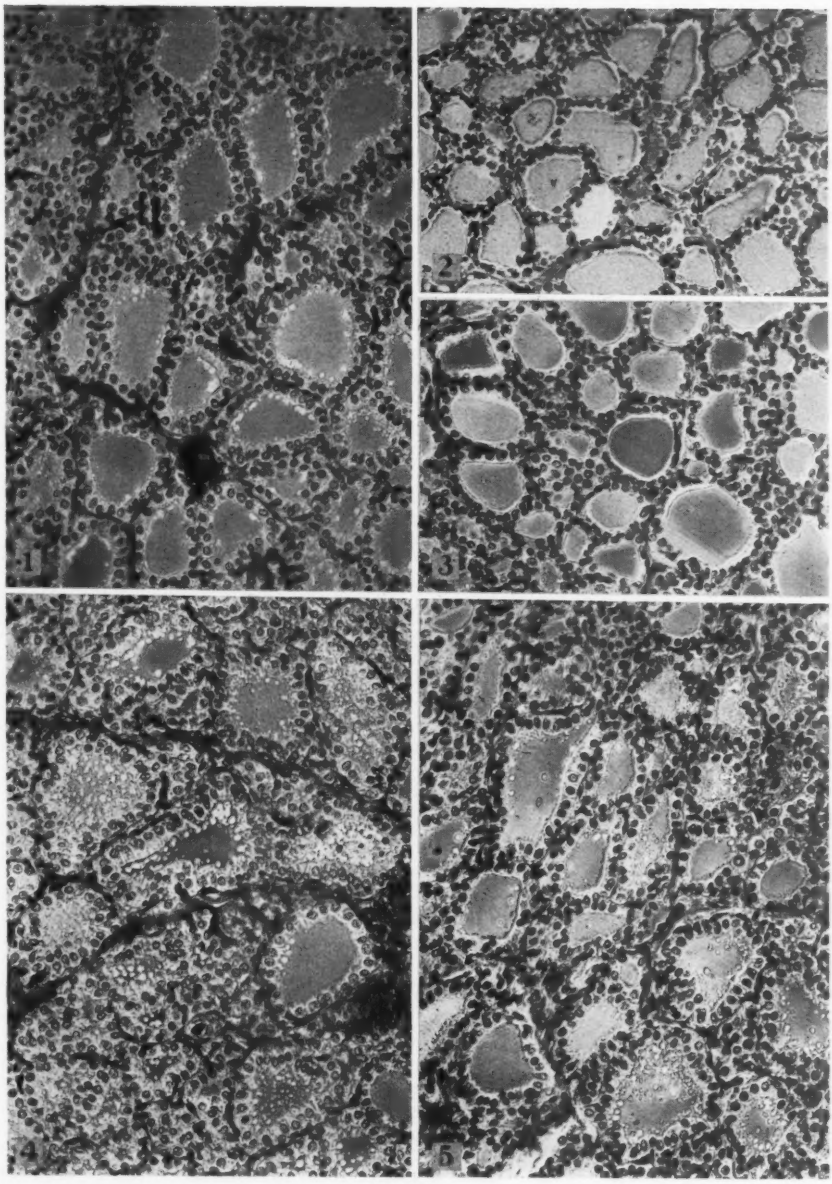


PLATE I

The enlarged and highly active parathyroids of normal members of normal-thyroidectomized pairs reported by Griesbach and Kennedy (1949) were not observed in the animals of this series.

## DISCUSSION

Blood from normal rats and thyroidectomized rats was permitted to flow into hypophysectomized partners through the vascular inosculation established between parabionts. It has been shown that the exchange of blood in each direction between members of such pairs is equal to approximately 10 times the blood volume per day (Van Dyke, Huff, and Evans 1948).

TABLE 3. AVERAGE ADRENAL WEIGHT AND AVERAGE SIZE OF ADRENAL CORTEX IN PARABIOTIC PAIRS

| Type of pair | No. of pairs | Weight left adrenal | Cortex size* | Adrenals compared SR‡ | | |
|---|---|---|---|---|---|---|
| | | | | | Weight | Cortex |
| | | mg. | | | | |
| N-N | 6 | 15.71 ± .42† | 14.56 ± .38 | H-H vs. N-N | 23.05 | 21.80 |
| T-T | 6 | 14.10 ± .61 | 13.15 ± .24 | H-N vs. H-H | 5.88 | 3.71 |
| H-H | 6 | 4.65 ± .25 | 4.75 ± .25 | H-T vs. H-H | 4.07 | 3.53 |
| N-T | 7 | 17.77 ± .73 | 16.26 ± .67 | T-N vs. N-N | 4.34 | 3.72 |
| N-H | 7 | 15.37 ± .91 | 13.94 ± .42 | | | |
| T-N | 7 | 19.01 ± .63 | 16.16 ± .20 | | | |
| T-H | 7 | 16.94 ± .87 | 15.14 ± .71 | | | |
| H-N | 7 | 6.70 ± .25 | 6.05 ± .24 | | | |
| H-T | 7 | 6.77 ± .46 | 6.50 ± .32 | | | |

* Size is expressed in planimeter units.

† SE (of mean) $= \frac{SD}{\sqrt{n-1}}$.

‡ SR = Significance ratio. The difference of the means was considered statistically significant if the SR was greater than 3.0.

Thus hypophysectomized animals receiving ten times their blood volume per day of blood from normal animals for 30 days, showed only minimal stimulation of their thyroids (plate 1, figs. 2 and 3). However hypophy-

## PLATE II

Photographs were taken with 4 mm. objective; magnification ×450, Mallory-Azan stain.

FIG. 1. Field from a section of anterior pituitary of a normal control rat (N-N). Note general appearance and distribution of cells.

FIG. 2. Field from a section of the pituitary of a thyroidectomized control rat (T-T). Note almost complete absence of acidophils. Note changes in basophils; numerical and size increase, multiple hyaline vacuoles prominent nucleoli and large Golgi apparatus.

FIG. 3. Field selected from the pituitary of thyroidectomized partner in T-N pair. Note that changes in basophile cells closely resemble those seen in figure 2. Eosinophiles are present.

FIG. 4. Field selected from the pituitary of a thyroidectomized partner in T-H pair. Note that many of the basophils still manifest typical thyroidectomy changes. Eosinophiles are present.

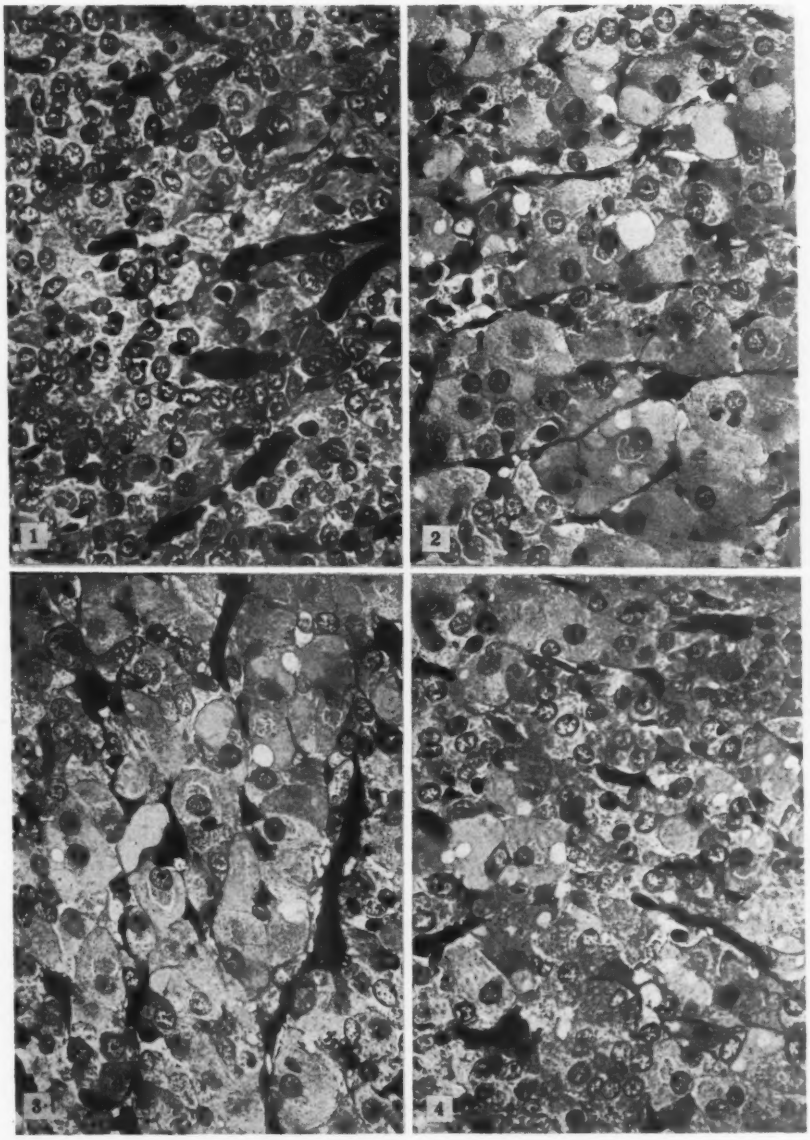


PLATE II

sectomized animals receiving the same amount of blood a day for the same period from thyroidectomized partners had thyroids equal in weight and histologic appearance to the normal. These findings indicate that the blood from thyroidectomized rats contained a much higher level of thyrotropic hormone than that from normal animals.

In single thyroidectomized animals one might expect still higher levels than in these pairs since in hypophysectomized-thyroidectomized parabionts the stimulated thyroid of the hypophysectomized partner is exerting an inhibitory influence on the pituitary of its thyroidectomized partner. Evidence of this inhibitory influence is shown by the incomplete thyroidectomy changes in the pituitaries of the thyroidectomized partners of thyroidectomized-hypophysectomized pairs.

The limitations of the method of parabiosis for measurements of the presence of humoral substances have been discussed by Huff, Trautman and Van Dyke (1950). The sensitivity of the "test partner" is one limiting factor. Thus when previous investigators have employed normal animals as the test partner for increased thyrotropic hormone, there was no easily recognizable thyroid stimulation. However, when hypophysectomized animals were used as test partners the increase in thyrotropic hormone after thyroidectomy was clearly evidenced.

It will be remembered that the interesting observation was made that the adrenals of the thyroidectomized members in parabiosis with normal partners (T-N) were larger than the adrenals of the normal controls (N-N). It is known that the level of thyroxin influences adrenal size: Absence of thyroxin (thyroidectomy) results in a decreased adrenal weight (Smith, Greenwood and Foster 1927); an excess of thyroxin results in an increase in adrenal weight above normal (Hoskins, 1910, and Herring, 1917). The adrenals of the thyroidectomized-thyroidectomized pairs were smaller than the normal-normal controls as was to be expected. The adrenals of the normal members of N-T pairs, where the thyroid showed hyperplasia, were larger than in the normal-normal. However, the reason for significant increase in the size of the adrenals of the thyroidectomized member of thyroidectomized-normal pairs has not satisfactorily been explained. The presence of thyroidectomy changes in the basophils of the pituitaries of thyroidectomized partners of thyroidectomized-normal pairs indicates that these rats were still in a state of partial thyroxin deficiency. Therefore the amount of circulating thyroxin in these animals could not have been sufficient to cause adrenal hypertrophy. The pituitary of the normal partner (N-T), if it were producing excessive amounts of adrenocorticotrophic hormone, could not be considered the source for stimulation of the adrenals of the thyroidectomized member, as a parabiont achieves only an extremely small fraction of the adrenocorticotrophic hormone level of its

partner (approximately 1/85, Van Dyke, Simpson, Li and Evans, 1950). The most likely explanation at present for increased adrenal size in thyroidectomized partners of T-N pairs is an excess of ACTH resulting from the changes induced in the pituitary by thyroidectomy.

## SUMMARY

The increase in thyrotropic hormone content of the thyroidectomized animals blood was clearly demonstrated in the response of the thyroid of the hypophysectomized partner by uniting hypophysectomized animals parabiotically with thyroidectomized partners in one case and normal partners in another case.

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# STEROID EFFECTS ON HUMAN LEUKOCYTES

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THE mechanism and site of the lytic and depressant action of adrenocortical compounds on lymphoid, connective and dermal tissues has not yet been elucidated (Dougherty and White, 1945; Heilman, 1945; Baker *et al.*, 1948; Baker and Whitaker, 1948; Valentine, Craddock and Lawrence, 1948; Hechter and Johnson, 1949; Schrek, 1949; Creditor *et al.*, 1950; Feldman, 1950; Meier, Schuler and Desaulles, 1950; Spain, Molomut and Haber, 1950; Cornman, 1951; Shapiro, Taylor and Taubenhaus, 1951). The present study was planned to ascertain morphologically the locus of this action at the cellular level and to determine whether adrenocortical reagents act upon enzyme systems within the cytoplasm or at the surface of the cell.

## MATERIALS AND METHODS

Supravital slide preparations were utilized. Janus green and neutral red, 1:1,000 in Locke's medium, were added to human blood to give a final dilution of 1:10,000. 0.2 ml. of steroid was shaken gently with 0.3 ml. of a) dyed blood, b) a mixture of dyed blood and enzyme poison, and c) a mixture of dyed blood and surface active compound. A drop of the final mixture was placed on a clean slide and covered with a cover slip which was then ringed with melted paraffin. In some experiments steroid was added to blood or the blood mixtures first and the dyes added later.

The materials consisted of the following:

White cells of human heparinized blood (0.1 ml. of Liquaemin[1] to 1 ml. of whole blood.)

Steroids: Lipo-Adrenal Cortex[2]; estrogens[3,4]; progesterone[4]; testosterone[4]; desoxycorticosterone (DOCA)[5].

Steroid controls: cotton seed oil containing 0.5% chlorbutanol[2]; cholesterol; olive oil.

Enzyme poisons: Potassium cyanide, sodium azide and maleic acid, final dilutions in blood M/500, M/500 and M/200 respectively.

Surface active compounds: calcium, as a 1.5% aqueous solution of $CaCl_2$ final con-

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[1] Roche-Organon, Inc. New Jersey.

[2] Kindly supplied by Dr. William J. Haines of the Upjohn Company, Kalamazoo, Michigan.

[3] Stilbestrol, 1 mg. per ml. Burroughs-Wellcome, England.

[4] Oestropur, 10,000 units per ml.; Luteospur, 5 mg. per ml.; Homosteron, 10 mg. per ml. These reagents were kindly supplied by Dr. Schindle of Teva, Israel.

[5] DOCA, 2 mg. per ml. NV Organon OSS, Holland.

centrations between 15 to 40 mg. %; polylysine[6], as a 1:1,000 aqueous solution, final dilutions between 75 to 500 μg./ml.; lactalbumin, as a 0.2% and 2% solution in Locke's medium, final concentrations of 0.1% and 1% respectively.

Control preparations were prepared and examined at the same time as the experimental slides. Controls consisted of a) dyed blood plus Locke's medium, b) dyed blood plus steroid control, c) dyed blood plus enzyme poison or surface active compound.

## RESULTS

Lipo-Adrenal Cortex produced two distinct effects in all the cell types studied: a) "bubbling" activity, and b) decolorization of mito-chondria and cytoplasmic vacuoles. Table 1 contains in summary form the data of all experiments.

TABLE 1. REACTIONS OF LEUKOCYTES, TO STEROIDS, ENZYME POISONS, SURFACE ACTIVE COMPOUNDS AND CONTROL MATERIALS

| Reagent | Bubbling | Decolorization mitochondria and vacuoles | Cell death | Locomotion |
|---|---|---|---|---|
| Lipo-Adrenal Cortex | + | 30–60 minutes | + | 0 |
| Lipo-Adrenal Cortex KCN, sod. azide or maleic acid | + | 1– 3 minutes | + | 0 |
| Lipo-Adrenal Cortex Calcium polylysine or lactalbumin | + | 30–60 minutes | + | 0 |
| Estrogen | 0 | 30–60 minutes | + | 0 |
| Testosterone | 0 | 30–60 minutes | + | 0 |
| Progesterone | 0 | 30–60 minutes | + | 0 |
| DOCA | 0 | 0 | 0 | + |
| Cholesterol | 0 | 0 | 0 | + |
| KCN M/500 | 0 | 1– 3 minutes | 0 | 0 |
| Sodium azide M/500 | 0 | 1– 3 minutes | + | 0 |
| Maleic acid M/200 | 0 | 0 | 0 | 0 |
| Calcium polylysine or lactalbumin | 0 | 0 | 0 | + |
| Cotton seed oil | 0 | 0 | 0 | + |
| Olive oil | 0 | 0 | 0 | + |
| Whole blood and dyes | 0 | 0 | 0 | + |

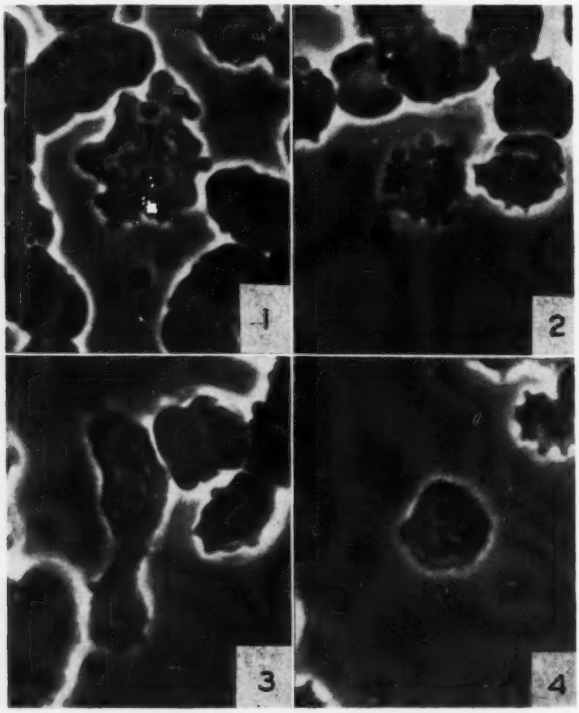
### a) *"Bubbling" activity* (Figs. 1 and 2)

Within 10 to 15 minutes after preparation of the slides, the majority of neutrophiles had rounded up and showed an intense "bubbling" of the cytoplasm. At several points on the periphery of the cell, pseudopods with or without cytoplasmic granules, suddenly formed. Some of these flattened down immediately and again formed pseudopods. Others slowly enlarged and broke away from their parent cell. At their former site, other pseudopods developed. At no time was locomotion of cells ever seen and the cyto-

[6] Obtained from Dr. E. Katchalsky of the Weizmann Institute for Science, Rehovoth, Israel. Polylysine is a polymer of the amino-acid lysine with a strong positive charge. It causes agglutination of red and white blood cells, apparently by altering the cell surface charge.

plasmic activity was completely different in "bubbling" neutrophiles from that seen in motile cells.

The "bubbling" activity continued for 20 to 30 minutes. During this time the neutrophiles either swelled slowly and disintegrated or formed a small round cell with serrated outline and gradually disappeared. If the cell became enlarged, the nucleus, mitochondria and vacuoles also increased in size.




EXPLANATION OF FIGURES

All photographs were taken through a Spencer phase microscope. Magnification, 900×. Enlarged three times.

FIG. 1. "Bubbling" neutrophile in the presence of Lipo-Adrenal Cortex.

FIG. 2. "Bubbling" lymphocyte in the presence of Lipo-Adrenal Cortex.

FIG. 3. Motile neutrophile in the presence of cotton seed oil.

FIG. 4. Lymphocyte in the presence of cotton seed oil.

All neutrophiles did not show this activity. Though actual counts were not made, it was estimated that 60 to 80% of the cells exhibited the phenomenon.

Lymphocytes, monocytes and eosinophiles of whole blood displayed similar "bubbling" activity, though not so intensely as did the neutrophiles. The number of pseudopods formed in these elements was fewer, the process slower, and breaking away was less frequently seen. These cell types also disintegrated after swelling.

Lipo-Adrenal Cortex caused "bubbling" of white blood cells in the presence of the enzyme poisons and the surface active compounds.

Estrogens, testosterone and progesterone caused no "bubbling" in leukocytes but damaged all cell types. DOCA, cholesterol, cotton seed oil and olive oil had no apparent effect upon the white blood cells.

In control preparations (Figs. 3 and 4), neutrophiles, lymphocytes, monocytes and eosinophiles exhibited all the well-known features already described by numerous investigators using supravital techniques (Cappell, 1929; Cunningham and Tompkins, 1930). Locomotion was always seen in the majority of neutrophiles. Very rarely one or two pseudopods were observed in an occasional cell of an entire preparation.

b) *Decolorization of mitochondria and cytoplasmic vacuoles*

Lipo-Adrenal Cortex decolorized within 30 to 60 minutes the mitochondria stained by janus green and the cytoplasmic vacuoles colored by neutral red in all leukocytic types. After removal of the hormone by alternate centrifugation and washing in Locke's medium, the mitochondria and vacuoles remained unstained. When Lipo-Adrenal Cortex and blood were first shaken together and the dyes added later, mitochondria and vacuoles were faintly stained for a short and variable period and finally lost their stain.

Estrogens, testosterone and progesterone similarly decolorized the mitochondria and cytoplasmic vacuoles. DOCA, cholesterol, cotton seed oil and olive oil were without visible effect.

Potassium cyanide and sodium azide decolorized within a few minutes the stained mitochondria and cytoplasmic vacuoles. Removal of potassium cyanide by alternate centrifugation and washing resulted in the reappearance of colored mitochondria and vacuoles (Brenner, 1949), although the hue was less intense than before the addition of potassium cyanide. Removal of sodium azide was not followed by the reappearance of color in the mitochondria and vacuoles. Maleic acid had no effect on either mitochondria or vacuoles.

The presence of calcium, polylysine or lactalbumin did not prevent the decolorization by Lipo-Adrenal Cortex of mitochondria and cytoplasmic vacuoles.

## COMMENT

The intense "bubbling" activity which occurred in the presence of Lipo-Adrenal Cortex was initially regarded as a morphological expression of the presence and utilization of increased amounts of energy, released by the catabolic action of the hormone. It was further supposed that after rapid utilization of the energy the enzymes associated with mitochondria and vacuoles were no longer able to maintain janus green and neutral red in an oxidized colored state. Disintegration was the final event.

The same "bubbling" activity, however, was observed in white cells in the presence of both Lipo-Adrenal Cortex and enzyme poisons. Despite the fact that potassium cyanide and sodium azide inhibited oxidation-reduction enzyme systems (Brenner, 1949; McShan, 1950) as visualized by the rapid destaining of mitochondria and vacuoles, "bubbling" occurred. The cytoplasmic activity was therefore probably not a result of increased energy production and utilization, although it is possible that mitochondrial and vacuolar enzymes are not related to pseudopod formation.

The significance and cause of cytoplasmic "bubbling" is obscure. It is probable that the rapid formation of pseudopods is similar to the shedding phenomena described by Dougherty and White (1945). Williamson (1950) has recently reported on and reviewed this subject and suggested that budding is most likely an activity of normal lymphocytes.

The site of lethal action of Lipo-Adrenal Cortex was not disclosed by the present investigation. The hormone did not specifically inhibit the staining of mitochondria and vacuoles as did potassium cyanide and sodium azide. When decolorization occurred the cells were obviously damaged and in the process of disintegration. Similar destaining occurred in cells of control slides which were injured during preparation. However, in the presence of Lipo-Adrenal Cortex the majority of white blood cells were destroyed within an hour, while in control mixtures the majority of leukocytes appeared still viable at the end of this period.

Lipo-Adrenal Cortex, moreover, did not appear to exert its action at the cell surface. In the presence of both Lipo-Adrenal Cortex and high concentrations of calcium ions, polylysine and lactalbumin, white blood cells reacted the same as in the absence of these compounds. Calcium, polylysine and proteins have been shown to alter the surface of macromolecules and a variety of cells (Abramson, Gorin and Ponder, 1940; Chambers, 1940; Feldman, unpublished observations). Inasmuch as white blood cells were not protected from the toxic effect of Lipo-Adrenal Cortex by the surface active compounds, it was assumed that either a) the hormone's effect did not take place at the cell surface; b) the surface active compounds did not alter white blood cells; or c) the dosage of the hormone used was excessive.

Estrogens, progesterone and testosterone also damaged and destroyed leukocytes, in much the same manner as Lipo-Adrenal Cortex but without causing cytoplasmic "bubbling." There are several studies which indicate that estrogens and testosterone are lympholytic and depressant for bone marrow (Selye, Harrow and Collip, 1936; Schacher, Browne and Selye, 1937; Chiodi, 1940; Reinhardt and Wainman, 1942; Crafts, 1948). It appears that a variety of steroids, at least in high concentrations, are lethal for white blood cells.

## SUMMARY

In the presence of Lipo-Adrenal Cortex neutrophiles, lymphocytes, monocytes and eosinophiles exhibited intense cytoplasmic "bubbling." This was followed by gradual decolorization of previously stained mitochondria and cytoplasmic vacuoles, and by cellular disintegration. Potassium cyanide and sodium azide caused rapid destaining of mitochondria and vacuoles but without cytoplasmic "bubbling." Surface active compounds did not protect leukocytes from the lethal action of Lipo-Adrenal Cortex. Estrogens, progesterone and testosterone also destroyed the white elements of human blood but in a somewhat different manner.

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# NOTES AND COMMENTS

## SECRETION RATE OF THYROID HORMONE IN WHITE LEGHORN CASTRATES[1]

The daily secretion rate of thyroid hormone has been studied in a number of species of animals and under different environmental conditions. Except for one report (Schultze and Turner, 1945), the secretion rate has not previously been determined in any castrate animals. It was thought profitable, therefore, to make such a determination as a part of a larger program in order to discover whether the absence of androgen affects the secretion rate of thyroid hormone.

The method employed for the determination of the thyroid secretion rate was essentially the same as that first developed by Dempsey and Astwood (1943). Male White Leghorn cockerels were placed on a diet of Purina Growena chicken feed containing 0.1% propylthiouracil[2] when they were 22 days old. At 24 days of age the birds were castrated and carefully allotted to series according to body weight and comb size. Injections of thyroxine at levels of 5.25 μg. per day, 10.50 μg. per day, and 15.75 μg. per day were begun the following day when the birds were 25 days old. Half of the birds receiving each of these levels of thyroxine were also injected daily with 40 μg. of testosterone propionate.[3] This amount of androgen is very slightly in excess of what has been determined as a normal daily level of secretion for White Leghorn cockerels of this age (Breneman and Mason, 1951). The birds were autopsied when they were 40 days old.

The secretion rate of the castrates not injected with androgen was determined to be 10.2 μg. equivalent of D,L-thyroxine per bird per day or 2.8 μg. per 100 gms. of body weight. The secretion rate of the castrates receiving daily injections of androgen was determined to be 10.1 μg. per bird per day or 2.7 μg. per 100 gms. of body weight per day. The data for these calculations are presented in Table 1.

The secretion rate of non-castrated White Leghorn cockerels has also been determined for this age range. The value obtained was 10.3 μg. per bird per day or 2.6 μg. per 100 gms. of body weight per day (Table 1).

It is thus apparent that over the period described in this experiment and at this age the secretion rate of thyroid hormone in the cockerel is not affected by the absence of the gonads, nor by the addition of androgen to capons at a slightly higher than physiological level. This is in contrast to the suggestive evidence of Schultze and Turner that less thyroid hormone was secreted by capons than by normal cockerels. It would be of interest to repeat the experiment on older birds which had been castrated for a longer period of time, since unpublished data from our laboratory (Breneman, personal communication) indicate that the thyroid weight of birds castrated at 5 days of age and carried to 120 days or older is less than that of control males of the same age.

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[1] Contribution No. 492 from the Department of Zoology, Indiana University.

[2] The propylthiouracil (6-n-propyl-2-thiouracil) was kindly supplied by Lederle Laboratories, Pearl River, New York.

[3] The testosterone propionate (Oreton) was kindly supplied by Schering Corporation, Bloomfield, New Jersey.

TABLE 1. THYROID SECRETION RATES OF NORMAL AND CASTRATE COCKERELS

| No. | D,L-thyroxine μg./bird/day | Body Wt. gms. | Thyroid Wt. mgs. | Thyroid Wt. mgs./100 gms. | Secretion Rate |
|---|---|---|---|---|---|
| | | Capon | | | |
| 12 | 0 | 362 | 23.8 | 6.57 | 10.2 μg./bird day |
| | | Capon +0.1% Thiouracil | | | |
| 10 | 0 | 305 | 88.6 | 29.05 | 2.8 μg./100 gms. body wt. |
| 13 | 5.25 | 339 | 74.5 | 21.98 | |
| 7 | 10.50 | 351 | 19.6 | 5.58 | |
| 9 | 15.75 | 336 | 11.9 | 3.54 | |
| | | Capon +T.P. | | | |
| 13 | 0 | 373 | 25.6 | 6.86 | 10.1 μg./bird/day |
| | | Capon +0.1% Thiouracil +T.P. | | | |
| 10 | 0 | 303 | 84.6 | 27.92 | 2.7 μg./100 gms. body wt. |
| 15 | 5.25 | 356 | 85.9 | 24.13 | |
| 12 | 10.50 | 360 | 18.8 | 5.22 | |
| 14 | 15.75 | 348 | 11.2 | 3.22 | |
| | | Male | | | |
| 10 | 0 | 391 | 24.5 | 6.27 | 10.3 μg./bird/day |
| | | Male +0.1% Thiouracil | | | |
| 13 | 0 | 342 | 108.7 | 31.78 | 2.6 μg./100 gms. body wt. |
| 12 | 8 | 379 | 36.6 | 9.66 | |
| 12 | 12 | 356 | 13.6 | 3.82 | |

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